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**INVESTIGATING THE GENE REGULATORY NETWORK UNDERLYING
CAUDAL HINDBRAIN SPECIFICATION IN EMBRYONIC ZEBRAFISH**

A Dissertation Presented

By

Priyanjali Ghosh

Submitted to the Faculty of the

University of Massachusetts Graduate School of Biomedical Sciences, Worcester

in partial fulfillment of the requirements for the degree of

DOCTOR OF PHILOSOPHY

June 13, 2018

Department of Biochemistry and Molecular Pharmacology

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This work was undertaken in the Graduate School of Biomedical Sciences

Department of Biochemistry and Molecular Pharmacology

Under the mentorship of

Charles G. Sagerström, PhD, Thesis Advisor

Michael M. Francis, PhD Member of Committee

Lawrence J. Hayward, MD, PhD, Member of Committee

Scot A. Wolfe, PhD, Member of Committee

Sarah McMenamin, PhD, External Member of Committee

Alonzo H. Ross, PhD, Chair of Committee

Mary Ellen Lane, PhD

Dean of the Graduate School of Biomedical Sciences

June 13, 2018

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Finally, to my family; nothing could possibly begin to describe the immense gratitude I have towards you all. None of this would have been possible without your unwavering love.

ABSTRACT

To understand the gene regulatory network (GRN) governing caudal hindbrain formation in embryonic zebrafish, several early expressed factors have been manipulated, and multiple genetic mutants have been characterized. Such analyses have identified morphogens such as Retinoic Acid (RA) and Fibroblast growth factors (FGFs), as well as transcription factors like *hoxb1b*, *hoxb1a*, *hnf1ba*, and *valentino* as being required for rhombomere (r) r4-r6 formation in zebrafish. Considering that the caudal hindbrain is relatively complex – for instance, unique sets of neurons are formed in each rhombomere segment – it is likely that additional essential genes remain to be identified and integrated into the caudal hindbrain GRN.

Our results reveal that r4 gene expression is unaffected by the individual loss of *hoxb1b*, *hoxb1a* or RA, but is under the combinatorial regulation of RA together with *hoxb1b*. In contrast, r5/r6 gene expression is dependent on RA, FGF, *hnf1ba* and *valentino* – as individual loss of these factors abolishes r5/r6 gene expression. Analysis of six mutant lines (*gas6*, *gbx1*, *sall4*, *eglf6*, *celf2*, and *greb1l*) did not reveal rhombomere or neuronal defects, but transcriptome analysis of one line (*gas6* mutant) identified expression changes for genes involved in several developmental processes – suggesting that these genes may have subtle roles in hindbrain development.

We conclude that r4-r6 formation is relatively robust, such that very few genes are absolutely required for this process. However, there are mechanistic differences in r4 versus r5/r6, such that no single factor is required for r4 development while several genes are individually required for r5/r6 formation.

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PREFACE

Sections from the following articles appear in whole or part in the thesis:

Ghosh P, Maurer JM, Sagerström CG. Analysis of novel caudal hindbrain genes reveals different regulatory logic for gene expression in rhombomere 4 versus 5/6 in embryonic zebrafish. *Neural Development (Research article under revision)*

Ghosh P, Sagerström CG. Developing roles for Hox proteins in hindbrain gene regulatory networks. *Int J Dev Biol. (Review article accepted)*

CHAPTER I: INTRODUCTION

Development of the vertebrate hindbrain

The organization and development of the hindbrain is evolutionarily conserved across all jawed vertebrates. During the early stages of vertebrate embryogenesis, the hindbrain appears as a featureless sheet of cells. Once the neural tube closes; cells in the presumptive hindbrain are sorted and segregated into seven molecularly and neuroanatomically unique segments called rhombomeres (r1-r7). Rhombomere segmentation creates regional diversity along the anterior-posterior (A-P) axis of the hindbrain and promotes proper patterning of the neural circuitry¹⁻³. Thus, each rhombomere represents a unique set of cell fates and is the source of distinctive neural progenitors that ultimately differentiate into segment-specific neurons. In particular, several rhombomeres contribute neurons to the cranial nerves, such that trigeminal neurons (nV) form in r2 and r3, facial neurons (nVII) in r4, abducens neurons (nVI) in r5 and r6, and vagal neurons (nX) in the caudal-most portion of the hindbrain. Additionally, reticulospinal neurons arise in a rhombomere-specific pattern with Mauthner neurons, which are particularly prominent in aquatic species, forming in r4 (Figure 1.1)⁴⁻⁶. Via these classes of neurons, the hindbrain is responsible for regulating processes such as breathing, heartbeat, circulation and wakefulness, as well as to ensure innervation of the face, head, and neck. Furthermore, the cerebellum arises from the dorsal most region of r1 and plays an essential role in motor control, as well as some cognitive functions^{2,3}. Hence, proper hindbrain segmentation is essential for correct fate

specification and appropriate anatomical positioning of key types of neurons and neural structures. Disruptions in these processes lead to incomplete neural circuits and abnormal neural function, and such defects have been linked to neurological disorders like autism ^{7–9}. Thus, we see that the hindbrain is a coordination center for motor activity and complex physiological processes, making it crucial to understand the regulatory mechanisms that govern hindbrain formation.

The current understanding of hindbrain development has been postulated by studying various model organisms. In the next few sections, we will discuss the role of some of the key factors that control hindbrain development, with a specific focus on our model organism of choice *Danio rerio* — the zebrafish.

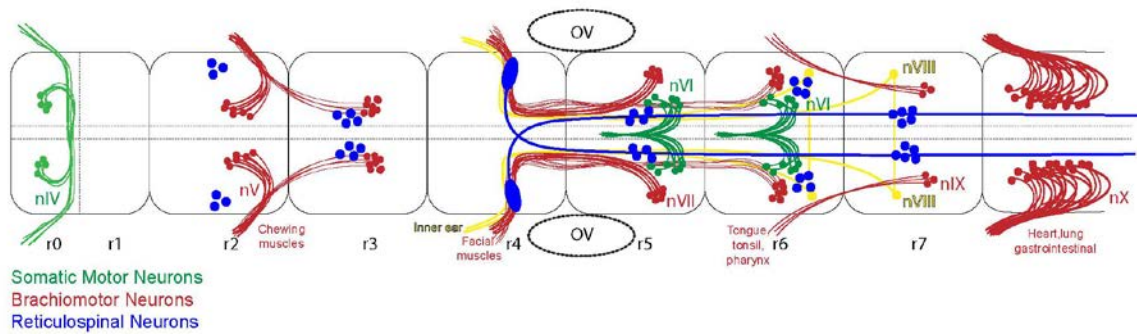


Figure 1.1 Neural Circuit in the zebrafish hindbrain

Neuronal cell bodies are born in specific rhombomeres and they follow a well-defined migratory pathway to their final destinations within the hindbrain. Facial motor neurons (red-nVII) are born in r4 from where the cell bodies undergo a characteristic rostro-caudal tangential migration to finally arrive at their destinations in r6 and r7. r4 is also where the Mauthner Neurons (blue) form. The abducens are born in r5 and r6 and move laterally and dorsally to settle in r5/6 (nVI-green).

Retinoic acid and fibroblast growth factors—the posteriorizing signals in the vertebrate hindbrain

During gastrulation, signals from the underlying mesoderm initiate the anteroposterior patterning of the neuroectoderm. The prevailing two-signal model of how this patterning is established, was first proposed by Pieter Nieuwkoop in 1952, and it suggests that regionalization of the neuroectoderm occurs through “Activation” and “Transformation”. Initially, the neural tube is induced to acquire anterior fates through “activating” signals such as *noggin* and *chordin*. Subsequently, graded “transforming” signals convert the neural tube into more posterior tissue. Such transforming signals include endogenous retinoic acid (RA) and fibroblast growth factors (FGFs) ^{10–12}.

Retinoic Acid

Retinoic acid is a small lipophilic morphogen that is a derivative of vitamin A or retinol. Retinol is converted to retinaldehyde through oxidation by alcohol dehydrogenases. This is followed by the conversion of retinaldehyde to RA by retinaldehyde dehydrogenases (*raldh2*). In the zebrafish (like most vertebrates), RA is synthesized in the posterior paraxial presomatic mesoderm. From there it diffuses throughout the neural tube and is detected in the hindbrain as early as 6 hours post fertilization (hpf) ^{13–21}. Subsequently, RA is degraded by a subclass of Cytochrome P450 enzymes called Cyp26 enzymes. In the zebrafish there are three Cyp26 (Cyp26a1, Cyp26b1, Cyp26c1) enzymes which are expressed primarily in the anterior neuroepithelium (presumptive r2-4) towards the end of

gastrulation (8-10 hpf). Over the course of development (12-20 hpf), the anterior boundary of these *cyp26* genes extend posteriorly to r6. This varying temporal and spatial expression pattern of the Cyp26 enzymes creates an oxidative sink for RA degradation forming a gradient wherein the caudal hindbrain is exposed to higher concentrations of RA (Figure 1. 2) ^{15,17-19,22-24}. In the hindbrain, RA binds to and activates a heterodimeric complex of RA receptors (RAR α , β , γ) and retinoic X receptors (RXR α , β , γ). These receptors are expressed from r4 to the anterior spinal cord from the gastrula stage to the end of segmentation (5-24 hpf) ²⁵. The RA-RAR/RXR complex enters the nucleus and targets genomic regulatory sequences known as RA response elements (RAREs) ^{16,18,24,26-29} resulting in the activation of signaling cascades that regulate the expression of several genes within the hindbrain.

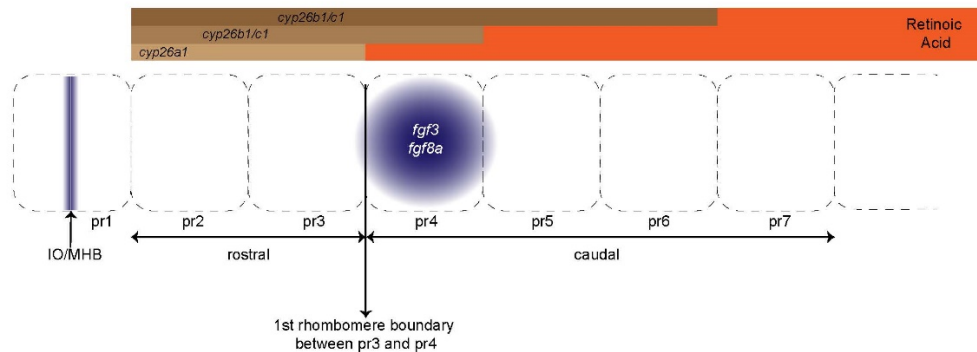


Figure 1.2 Division of the pre-rhombomeric hindbrain into rostral and caudal regions.

Retinoic Acid (orange bar) is expressed in the posterior paraxial presomatic mesoderm and spreads anteriorly. The three Cyp26 enzymes (brown bars) are expressed in the rostral/anterior regions of the hindbrain and degrade RA. Cyp26 enzymes are expressed in different regions of the anterior hindbrain during the course of development. These complementary expression domains of RA and Cyp26 enzymes set up the rostral and caudal regions of the hindbrain such that the first rhombomere boundary forms between the presumptive rhombomere (pr)3 and pr4. FGF signaling sources include the isthmic organizer/mid-hindbrain boundary (IO/MHB) and pr4. *fgf3* and *fgf8a* in the pr4 creates a signaling center which further defines the future r4 region—the first rhombomere to be formed

Fibroblast growth factors

In addition to RA, FGFs are another group of secreted morphogens that can convert anterior neural tissue to posterior neural fates. FGFs are expressed in the undifferentiated mesendoderm as early as the blastula stage (2-5 hpf) and are then found in the midbrain and hindbrain region starting at the gastrula stage (5-10 hpf) ³⁰⁻³². In zebrafish, the primary sources of FGF signaling in the neural tube includes the isthmus organizer (IO- region between midbrain and r1)/mid-hindbrain boundary (MHB) and r4. The two FGFs that establish the signaling center in the presumptive r4 include *fgf3* and *fgf8a* and they are detected around 7-9 hpf (Figure 1.2) ^{21,32,33}. FGF signaling is transmitted and regulated through corresponding FGF receptors (*fgfr1*, *fgfr2*, *fgfr3*, and *fgfr4* in zebrafish) which are expressed throughout the zebrafish neural tube. The association of the FGF ligand to its receptor leads to the activation of the canonical ERK signaling pathway which in turn promotes the expression of genes controlling cell proliferation and differentiation ^{34,35}.

The expression of RA and FGFs starts the early subdivision in the A-P axis. In fact, the complementary expression domains of zebrafish *cyp26* genes and RA results in the appearance of the first rhombomere boundaries between r3 and r4, and r4 and r5. Furthermore, the establishment of the FGF signaling center defines the r4 territory which is now the first rhombomere to be formed (Figure 1.2) ^{2,31,36}. In almost all model vertebrate organisms (chick, frog, mouse,

fish), disruption of RA and FGF signaling leads to severe defects in caudal hindbrain formation. For example, disruption of RA signaling causes enlargement of anterior rhombomeres, abnormal formation of r4-7, mispatterning of motor neurons, and loss of otic vesicles. Similar defects in the posterior rhombomeres and caudal hindbrain neurons are seen due to improper FGF signaling^{13,14,17,19–21,24,32,37–40}. Thus, RA and FGF signaling play a critical role in initiating the rostro-caudal division of the hindbrain. Additionally, these caudalising morphogens also trigger the expression of several transcription factors (TFs) in a spatially restricted manner resulting in further specification and partitioning of the hindbrain into its discrete rhombomeric compartments. Moving forward, we will focus our discussion on how these caudalising morphogens and the associated TFs develop and maintain the identity of the posterior rhombomeres (r4-6) in the hindbrain.

Transcription factors specifying the caudal hindbrain in vertebrates

Segmentation and specification of the hindbrain is orchestrated by a relatively conserved web of interacting genes, referred to as a gene regulatory network (GRN). Some of the key inductive signals within the caudal hindbrain GRN include TFs like genes in the *Hox* cluster, *Vhnf1/Hnf1b*, *Kreisler/MafB/valentino*, and *Krox20/Egr2* (Figure 1.3). In the next few sections, we will discuss how these GRN TFs govern the formation of the caudal hindbrain (r4-6) in vertebrates.

The role of Hox genes in caudal hindbrain formation

Hox genes are a family of highly conserved homeodomain transcription factors that regulate axial patterning in most animal species ⁴¹. Most vertebrates have 39 *Hox* genes located in four chromosomal clusters and are classified into 13 paralog groups (PG). Teleost fish have undergone a major whole-genome duplication event and have also lost of a few genes over the course of evolution. As a result, zebrafish have 48 *hox* genes arranged in seven clusters ⁴². A hallmark of these *Hox* genes is collinearity—the linear arrangement of the *Hox* clusters on the chromosomes correspond to the spatial and temporal expression pattern of the genes ⁴¹. The role of *Hox* genes in specifying segment identity was first uncovered in *Drosophila* and an equivalent role was later confirmed in vertebrates ^{43,44}. Specifically, *Hox* genes from PG1-3 are expressed in nested domains along the developing vertebrate hindbrain and this “*Hox* code” controls segmentation and specification of rhombomeres ^{2,45}.

RAREs are present in the *Hox* clusters ⁴⁶ where RA promotes decondensation of otherwise compacted chromosomal chromatin, thereby permitting active transcription ^{47–49}. Thus RA induces the expression of the first *Hox* genes (*Hoxa1* in the mouse and *hoxb1b* in zebrafish) which are expressed during gastrulation and are transcribed in the caudal domain with their anterior limit at the future boundary between r3 and r4 ^{50–52}. Shortly thereafter, *Hoxb1* (*hoxb1a* in zebrafish) becomes expressed in a domain that coincides with the future r4 ^{53–55}. Accordingly, the PG1 *hox* genes (*Hoxa1/hoxb1b* and

Hoxb1/hoxb1a) are required for formation of r4, but they play distinct roles. For example, *Hoxa1/hoxb1b* mutants have a smaller r4 (as well as a larger r3), while *Hoxb1/hoxb1a* mutants have a mis-specified r4, where facial motor neurons fail to migrate out of r4 ^{49,56–64}. Specifically, *hoxb1a* mutant zebrafish also lack the r4-specific Mauthner neurons ^{62,64}. *Hoxb2* (*hoxb2a* in zebrafish) is expressed in r3-r5 and, in mice, mutations in this gene disrupt r4 specification such that formation of the facial motor neurons is disrupted ⁶⁵. However, it does not appear to affect the formation of other rhombomeres. In contrast, mutations in the murine *Hoxa2* (*hoxa2b* in zebrafish), which is expressed in r2-r5, does not affect segmentation or specification of the corresponding rhombomeres ^{66,67}. Notably, the corresponding zebrafish morphants of PG2 *hox* genes (*hoxb2a* and *hoxa2b*) show mild defects in the second pharyngeal arch, but no severe defects in rhombomere formation ⁶⁸. Mutations in PG3 *hox* genes (particularly simultaneous loss of *Hoxa3* and *Hoxb3*) disrupt formation of the Abducens motor nucleus in r5 in mice ⁶⁹. In the zebrafish, *hoxa3* (r5/6) and *hoxb3* (r5 to spinal cord) are suggested to indirectly regulate the proper formation of pharyngeal arches 3-7 ⁷⁰. Thus, barring a few species-specific differences in function, *Hox* genes are essential for segmentation (*Hoxa1/hoxb1b*) and specification (*Hoxb1/hoxb1a*, *Hoxb2/hoxb2a*, *Hoxa3* and *Hoxb3*) of several rhombomeres in vertebrates (Figure 1.3).

After *Hox* genes were initially cloned, it became clear that they encode proteins containing a helix-turn-helix DNA binding motif – the homeobox ⁷¹ –

suggesting that they function as transcription factors to control gene expression⁷². However, subsequent detailed biochemical analyses revealed that Hox proteins have poor affinity and specificity for DNA, with most Hox proteins preferring to bind AT-rich sequences. Accordingly, Hox proteins function in complexes with other TFs that facilitate their binding to DNA and that ensure greater sequence selectivity^{73,74}. In particular, Hox proteins bind genomic DNA in complexes with members of the TALE (three amino acid loop extension) family of homeodomain proteins. Combining data from mouse and zebrafish, the TALE family includes at least four Pbx, four Meis and three Prep TFs that can interact with Hox proteins – where the Prep and Meis proteins are more closely related to each other than to Pbx⁷⁵. Many Hox proteins bind Pbx TFs using a short motif (YPWM) found N-terminal to the Hox homeodomain⁷⁶, while other Hox proteins bind members of the Prep/Meis family via N-terminal sequences in the Hox protein^{77,78}. Notably, Pbx and Prep/Meis form heterodimers, meaning that TALE TFs can be part of a Hox transcription complex not only by binding Hox proteins directly, but also via their interactions with each other. As a result, many Hox-controlled regulatory elements have been shown to be occupied by trimeric Prep/Meis: Pbx: Hox complexes^{52,79–84}. In particular, trimeric complexes containing the earliest expressed Hox proteins in the hindbrain (Hoxa1/Hoxb1b) are required for the initial expression of hindbrain-specific genes such as *Hoxb1/hoxb1a* and *Hoxb2/hoxb2a*.

Non-Hox transcription factors in the caudal hindbrain

Vhnf1/Hnf1b

Variant hepatocyte nuclear factor 1 (*Vhnf1*) or *hnf1ba* (in zebrafish) is a homeobox transcription factor that is expressed throughout the caudal hindbrain and anterior parts of the spinal cord. It has been shown that RA interacts with an 800bp RARE in the fourth intron of *hnf1ba* and drives its expression from the posterior hindbrain to presumptive r4/5 boundary^{17,36,85,86}. In zebrafish, *hnf1ba* denotes the future r5 and r6 territories within the hindbrain and disruptions in *hnf1ba* results in improper gene expression in r4-6. Additionally, in *hnf1ba* mutants, abducens motor neurons (nVI in r5 and r6) fail to develop properly and they also have an extra set of Mauthner neurons^{85,87}. While knockout mice for *Vhnf1* have been generated, they have not been studied in the context of hindbrain development. Moreover, in humans and mice, HNF1B has a role in liver and pancreas development and mutations in this gene are associated with diabetes and liver disease^{88–90}

MafB/Kreisler/valentino

Vhnf1/Hnf1b cooperates with FGF signaling to regulate the expression of the bZip transcription factor *MafB* (*Kreisler* in mouse) and (*valentino* in zebrafish)^{85,91}. *MafB/Kreisler/valentino* divides the presumptive r5/6 “proto-segment” into distinct r5 and r6 domains in a cell-autonomous manner and thus is required in establishing the boundary between r5 and r6 (Figure 1.3)⁹². Accordingly, *valentino* mutants lack specific rhombomere boundaries past r4, and r5/6 collapses into a smaller, poorly-defined r “X” domain. While the reticulospinal neurons are un-altered, the abducens neurons are lost and the otic vesicles are reduced in size. Similar segmentation, gene expression and inner ear defects are seen in the murine *Kreisler* mutants^{54,92,93}.

Krox20/Egr2

The zinc finger transcription factor *Krox20* (previously known as *Egr2*) is expressed in rhombomeres 3 and 5 in chick, mouse, and zebrafish. The expression of *Krox20* in r5 is initiated by inputs from *Vhnf1/Hnf1b*, *valentino/Kreisler*, and FGF signaling (Figures 1.3 and 1.4). In zebrafish (and chick) development, it has been demonstrated that FGF signaling initiates *krox20* in r5 by binding to and modulating the enhancer activity of the cis-regulatory elements B and C. Furthermore, *MafB*, mediates and maintains *krox20* expression in r5 by directly binding to element B. Once expressed, *krox20* also regulates its own expression by interacting with the autoregulatory element A. As for r3 expression of *krox20*, at least in the zebrafish, it has been suggested

that Irx and Meis proteins play a role in its regulation. During early stages of development, low doses of *hoxb1a* initiates the expression of *krox20*. With the subsequent definition of r3 and r4, the increased levels of *hoxb1a* will interact with element C and prevents the expansion of *krox20* into r4^{94–101}. In mice, *Krox20* is not required for initial delimitation of r3 and r5; however, it is required to maintain boundaries between odd and even rhombomeres. It does so by directly activating EphA4/Sek1 (a transmembrane receptor tyrosine kinase), a protein that is known to be involved in cell sorting and segregation^{94,102–104}. Additionally, in mouse, *Hoxb2*, *Hoxa2*, and *Hoxb3* are direct transcriptional targets of *Krox20* (Figure 1.3)^{102,103,105,106}. Mutant *Krox20* mice lack r5 and have facial motor neurons that migrate incorrectly through the neural tube. *Krox20* null mice eventually die within the first two weeks after birth^{94,107}. While zebrafish mutants for *krox20* have not been studied in the context of hindbrain formation and patterning, it has been suggested that *krox20* modulates Schwann cell mediated myelination¹⁰⁸.

The historical model describing the caudal hindbrain (r4-6) gene regulatory network in zebrafish

Thus far, we have broadly discussed some of the key factors that regulate caudal hindbrain development in vertebrates. In this section, we will focus on the historical model that describes the role of these key factors in the GRN governing caudal hindbrain (r4-6) formation in zebrafish (Figure 1.4).

Around 6 hpf, RA is detected in the posterior paraxial mesoderm from where it diffuses throughout the neural tube creating a spatial gradient with the caudal hindbrain being exposed to higher concentrations of RA.^{16–21} FGF signaling (*fgf3* and *fgf8a*) is detected as early as 8 hpf in the presumptive r4^{21,32,33}. Expression of these morphogens initiates the division of the hindbrain primordium into rostral and caudal regions. The subsequent expression of transcription factors (TFs) like *hoxb1b*, *hoxb1a*, *hnf1ba*, *valentino* and *krox20* further subdivides the caudal hindbrain into r4, 5 and 6. Expression levels of these factors have been manipulated to gain insight into how they interact with one another. With the discovery of several hindbrain specific genetic mutants, more information has been gathered about the role of these genes in the caudal hindbrain development. For example, loss of RA signaling results in posterior expansion of r4 genes like *hoxb1a*, and the loss of genes like *hoxb1b*, *hnf1ba* and *valentino*^{14,17,19}. A combined knockdown of *Fgf8* and *Fgf3* with morpholinos (MOs) leads to the loss of *krox20* (only in r5) and *valentino*^{21,33}. Mutations in the *hnf1ba* and *valentino* genes cause mis-patterning of r5 and 6 with the loss of *krox20* expression in r5 and posterior expansion of *hoxb1a* (only in *hnf1ba* mutants)^{85,93}. Germline mutants for *hoxb1b* have a smaller r4, with mis-patterned cranial motor neurons, and loss of Mauthner neuron formation. Similar neuronal defects are also seen in *hoxb1a* mutants⁶⁴. These results have contributed to the formulation of a gene regulatory network (GRN) underlying caudal hindbrain formation. The current understanding of the network shows RA initiating the expression of

hoxb1b (r4-7) and *hnf1ba* (r5/6) ¹⁷. *hoxb1b* turns on the expression of *hoxb1a* leading to the specification of r4 ^{52,64,109,110}. *hnf1ba* and *fgf3/8* co-operate to activate *valentino* expression in r5/6 ^{85,87}, and *valentino* and FGF signaling control expression of *krox20* in r5 ^{33,99}—thus defining the r5/6 domains of the hindbrain.

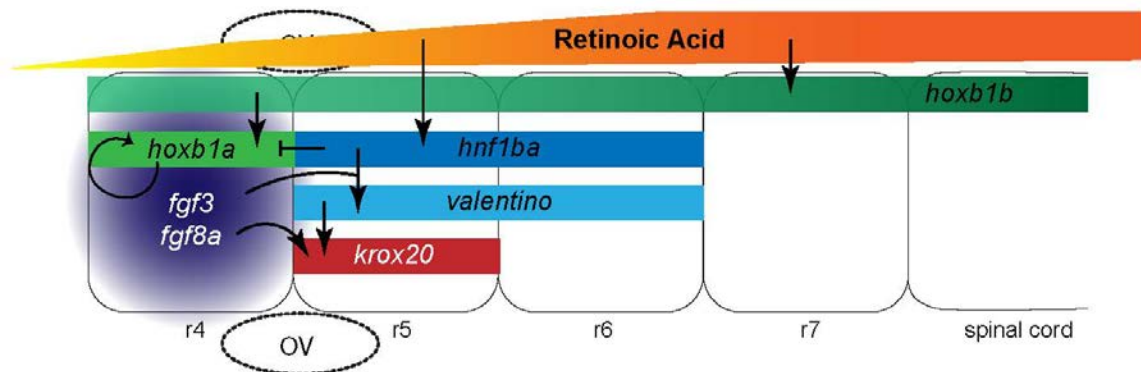


Figure 1.4 Historical model describing the caudal hindbrain (r4-6) gene regulatory network in zebrafish.

RA regulates the expression of *hoxb1b* (r4-7) and *hnf1ba* (r5/6). *hoxb1b* drives *hoxb1a* expression which can regulate its own expression in r4. *hnf1ba* co-operates with FGF signaling to turn on *valentino*. Both *valentino* and FGF signaling turn on *krox20* expression in r5. *hnf1ba* represses *hoxb1a* from expanding into r5/6.

Current understanding of the gene regulatory network governing caudal hindbrain (r4-6) formation is incomplete

The data supporting the proposed caudal hindbrain GRN were gathered by manipulating gene expression, and by studying germline mutants. In 1996, two large scale mutagenesis screens were performed using N-ethyl-N-nitrosourea (ENU) to identify genes involved in zebrafish development ^{111,112}. 70% of the mutants recovered from the screens showed extensive cell death and were discarded from further analysis. The remaining 30% showed developmental defects in the notochord, brain, skin, eye, jaw, gut, liver, heart etc. Mutants uncovered from these ENU screens demonstrate the role of several genes in a variety of developmental process, such as *fgf8a* ¹¹³, *pbx4* ¹¹⁴, and *valentino* ⁹³ which are required for proper hindbrain development in the zebrafish. While a large number of genes were mutated via ENU, only a few hundred genes have been identified, primarily due to the laborious nature of positional cloning. Furthermore, identified genes were cloned via a candidate-gene-approach—skewing the results towards previously characterized genes studied in other developmental processes ^{115–118}. The next tool to be used in forward genetics came in the form of retroviral mutagenesis. The Hopkins lab identified the Moloney murine leukemia retrovirus (MoMLV) as an effective mutagen. Once this virus is injected into zebrafish embryos, it integrates into the host's DNA leading to a loss or reduction in gene function-creating amorphic and hypomorphic mutants ¹¹⁹. Additionally, the viral DNA acts a molecular landmark

allowing for easier identification of the mutated gene ^{115,118,120}. Using this retroviral insertional scheme, the Hopkins lab performed a large scale mutagenesis screen and has cloned approximately 335 genes including hindbrain genes like *hnf1ba*, *pou5f3* and *mib1* ^{115–117,120,121}.

Although chemical and retroviral mutagenesis screens have shed light on a large number of genes regulating zebrafish development, these methods have several drawbacks. In both approaches, zebrafish embryos were visually screened for gross developmental defects between 24 hpf to 6 dpf (days post fertilization). As a result, mutations occurring during early stages of development were not detected ^{111,112,115,117}. Additionally, the presence of mutational “hot-spots” makes it difficult to extrapolate the extent to which the genome was saturated with these mutagens. Specifically, it has been estimated that the forward genetic screens in zebrafish reached only 25% (insertional screens) to 50% (ENU screens) saturation of the genome ¹¹⁹. Consequently, it is likely that a large number of genes involved in developmental processes remain unidentified.

With the release of the annotated zebrafish genome in 2001 ¹²², it became more desirable to knockout/inactivate a gene of interest and then hunt for a resulting phenotype. The TILLING (Targeted Induced Local Lesions) reverse genetics strategy has been employed to find genes of interest from a chemically mutated population ¹²³ and has identified mutations in the *krox20* ¹⁰⁸ and the *hoxb1b* ¹²⁴ alleles, among many others. Currently, antisense morpholinos (MOs), zinc finger nucleases (ZFNs), and transcription activator-like

effector nucleases (TALENs) are being used to knockdown and inactivate genes in the zebrafish ^{125–130}. In fact, our lab has utilized the ZFN and TALEN systems to generate germ-line mutants for *hoxb1b* and *hoxb1a* respectively ⁶⁴. As of 2013, disruptive mutations have been identified in only 38% of all protein coding genes—leaving room for more discovery ¹³¹. With the advent of the CRISPR/cas9 genome editing mechanism, it has become easier to create targeted and heritable mutations in genes of interest ^{129,132}. Currently, several labs are working on improving the CRISPR/Cas9 technique to increase the efficiency with which biallelic mutations are created and screened in the zebrafish ^{133–136}.

As forward genetics are limited by their saturation efficiency and reverse genetic screens are inherently biased towards a researcher's gene of interest, it seems likely that important regulators of r4-6 formation remain to be identified and that our current understanding of the GRN is indeed incomplete.

Approaches taken to expand the existing caudal hindbrain gene regulatory network

Despite extensive forward and reverse genetics approaches, the morphogens RA and FGF along with TFs *hoxb1b*, *hoxb1a*, *hnf1ba*, and *valentino* are the only known factors required for caudal hindbrain formation. This is surprising since GRNs associated with developmental processes (germ layer differentiation in sea urchin ¹³⁷, embryonic development in *c. elegans* ¹³⁸, pancreas formation ¹³⁹, mouse neural tube specification ¹⁴⁰ and zebrafish

endoderm formation ¹⁴¹) are extremely complicated and are riddled with feedback, feedforward, auto/cross regulatory interactions ¹³⁹—implying that there must be more genes present within the GRN that are likely to be required in caudal hindbrain formation. To identify additional components of the GRN, experiments such as yeast two hybrid studies (characterizing the mammalian *Hoxa1* interactome ¹⁴²), induction of *Hoxb1* and *Hoxa1* in murine embryonic stem (ES) cells ^{143–146}, MO-knockdown of *hoxb1b* and *hoxb1a* ^{109,147,148}, and overexpression of *hoxb1b* and *hoxb1a* ^{149,150} have been conducted. By virtue of being identified through these biochemical assays, these putative target genes can be positioned downstream of the *hox* genes. Although some of these genes may regulate developmental processes like the migration of neural crest cells, and neuronal patterning and differentiation ^{146,148}; to date, there is no evidence implicating them as indispensable regulators of r4-6 formation.

Investigating the gene regulatory network underlying caudal hindbrain specification: the rational and strategy behind this study

The intricate and precise formation of the caudal hindbrain and the ensuing establishment of the neural circuit within it is a manifestation of the its underlying GRN. Considering the complexities of this process, it is surprising that, despite a variety of approaches, only a few factors (RA, FGF, PG1 *hox* genes, *hnf1ba* and *valentino*) are considered the primary regulators of caudal hindbrain (r4-6) formation in the zebrafish. This leads us to speculate whether a

few genes are sufficient to form r4-6 or that prior analyses have overlooked critical genes that are necessary for the proper formation of the caudal hindbrain. Thus, the goal of this study was to identify novel regulators required for caudal hindbrain development and subsequently position them within the GRN governing caudal hindbrain formation.

In an effort to do so, we reasoned that it is highly probable that potential regulators are expressed in r4-6 at early stages of development—similar to the TFs *hoxb1b*, *hoxb1a*, *hnf1ba*, and *valentino*. To find such genes, we analyzed the gene expression data deposited in “The Zebrafish Information Network” (ZFIN) and curated a list of 107 genes that are expressed in r4, 5 and 6 during the first 24 hours of zebrafish development (discussed in detail in Chapter III). The majority (n=84) of these 107 genes remain uncharacterized (Appendix A and B), suggesting that they might be novel regulators of caudal hindbrain formation. To test this, we selected 22 candidate genes and assayed their expression patterns in mutants for *hoxb1b*, *hoxb1a*, *hnf1b*, and *valentino* via RNA in situ hybridization (ISH). We also investigated the effects of the morphogens RA and FGF on the expression of our candidate genes. Lastly, we also analyzed six germline (*gas6*, *gbx1*, *sall4*, *eglf6*, *celf2*, and *greb1l*) mutants for defects in hindbrain and neuronal patterning. Strikingly, our results show that genes expressed in r4 are not affected by the loss of *hoxb1b* or *hoxb1a*. Loss of RA and FGF signaling (except for *dusp2*, *dusp6*, *spry1*, *fgf3* and *fgf8*-which require FGF signaling) also does not affect r4 gene expression. Instead we find that all

candidate r4 genes are under the combinatorial regulation of RA and *hoxb1b*. Furthermore, these ISH analyses helped us identify additional r4 genes that are repressed by *hnf1ba*. Furthermore, we observe that *hoxb1a* (either directly or indirectly) represses the expression of *gbx1* in r4, revealing a novel relationship between *hoxb1a* and *gbx1*. In contrast to the complex gene regulation in r4, candidate r5/6 are under the regulation of RA, FGF, *hnf1ba* and *valentino*—whereby loss of any one of those four factors disrupts candidate r5/6 gene expression. Analysis of hindbrain and neuronal markers reveals that mutations in *gas6*, *gbx1*, *sall4*, *eglf6*, *celf2*, and *greb1l* are not sufficient to cause detectable developmental defects in the caudal hindbrain. Nevertheless, transcriptome analysis of *gas6* mutants shows that *gas6* is indeed involved in a variety of developmental processes, indicating that remaining candidate genes could also play a role in caudal hindbrain formation-albeit subtle.

Contribution of this work to the field

By successfully positioning 22 novel genes into the caudal hindbrain GRN, we demonstrate that gene regulation in r4 is robust with no single gene being essential, whereas r5/6 gene expression is susceptible to disruption of either RA, FGF, *hnf1ba* or *valentino* function. We also identify novel interactions between r4 and r5/6 genes—highlighting the importance of cross-talk between the two gene-sets in maintaining the specific molecular identity of each rhombomere. We have also generated two germline mutants for the genes *gas6* and *gbx1* using the

CRISPR/cas9 genome editing system. Transcriptome analysis of *gas6* mutants indicate that it may be involved in several important developmental processes. Overall, the analysis presented in this work demonstrates that hindbrain gene regulatory network is highly robust with only a few key genes being absolutely required for their integrity.

Thus, the results presented in this study have deepened and expanded the current understanding of how genes are regulated within the caudal hindbrain of the zebrafish.

CHAPTER II: METHODS AND MATERIALS

Zebrafish Care

Wildtype (WT) and mutant zebrafish embryos were collected through natural matings. All embryos were staged according to previously described morphological criteria ¹⁵¹. All zebrafish lines were raised in the University of Massachusetts Medical School Zebrafish Facility.

In situ hybridization

Embryos were collected at various timepoints between 11hpf and 24hpf and were fixed in 4% paraformaldehyde and stored in 100% methanol at -20°C. In situ hybridization (ISH) was performed as previously described and was followed by a color reaction using NBT/BCIP or INT/BCIP in 10% polyvinyl alcohol ¹⁵². Synthesis of RNA probes for the genes *dusp6*, *dusp2*, *krox20*, *hoxb1a*, *fgf3*, *fgf8* and *valentino* has been previously described ³⁵. 800-1000bp of coding sequence for the genes *pax2*, *spry1*, *hoxd4a*, *dm20*, *efnb2a*, *sall4*, *greb1l*, *egfl6*, *hoxb2a*, *engrailed1b*, *irx7*, *meis1a*, *tox3*, *sema3fb*, *mpz*, *gas6*, *hoxb3a*, *hoxa3*, *isl1/2*, *neurod6b*, *atoh1b*, *olig4* and *nr2f2* were cloned and used for probe synthesis. The *ccnjl*, *cefl2*, *col15a1b* and *gbx1* probes were purchased from the Zebrafish International Resource Center (ZIRC).

Immunostaining

For whole-mount immunostaining, embryos were fixed in 4% paraformaldehyde/8% sucrose/1x PBS. Fluorescent antibody staining was performed as described previously¹⁵³. Primary antibodies were used to detect Mauthner neurons (3A10; 1:100; Developmental Studies Hybridoma Bank [DSHB]), and Abducens motor neurons (mouse anti-Zn8; 1:1000; DSHB). The secondary antibody used was goat anti-mouse Alexa Fluor 488 (1:200; Molecular Probes A11001).

Imaging

Embryos between 11hpf and 19hpf were suspended in 3% methyl cellulose for imaging. Images were captured using a Leica M165 FC microscope equipped with a Leica DFC310 FX camera. 24 hpf, 48 hpf and 4 days post fertilization (dpf) old embryos were de-yolked and flat-mounted in 70% glycerol for imaging on bridged coverslips. Whole-mount embryos were imaged with a Nikon Eclipse E600 microscope equipped with a Nikon 20x Plan Fluor objective and flat-mounted embryos were imaged with a Zeiss Axiocam 503 color camera. Captured images were cropped and adjusted (limited to contrast and levels) in Adobe Photoshop.

Pharmacological treatments

A 250mM stock solution of SU5402 (a competitive inhibitor of the Fgf receptor tyrosine kinase; Calbiochem) and a 1M stock solution of 4(Diethylamino)-benzaldehyde (DEAB – small molecule inhibitor of RALDH enzyme involved in RA synthesis; Aldrich) was diluted in DMSO and stored in -20°C. To block RA signaling, embryos were soaked in 10uM DEAB starting at 4 hpf. The drug was never washed off and embryos were collected and fixed for ISH at 12hpf, 14hpf, 16hpf, 19hpf and 24 hpf. Similarly, to block FGF signaling, embryos were soaked in 50uM of SU5402 from 7hpf to 12hpf. After which, embryos were thoroughly rinsed in aquarium water ³² and allowed to develop till 12hpf, 14hpf, 16hpf, 19hpf and 24 hpf when they were collected and fixed for ISH.

Design and injection of single-strand guide RNAs for CRISPR/Cas9 mediated genome editing

gas6 (alleles um296, um297, um298 and um299) and *gbx1* (alleles um300 and um301) mutants were generated using the CRISPR (Clustered Regularly Interspaced Short Palindromic Repeat)/Cas9 genome editing system. Target sites for *gas6* (5'-ATGAGGGAGCTGGTGTGGAGC-3') and *gbx1* (5'-CCAGATAGT- TTCTACCCCCC-3') were determined using the CHOPCHOP web tool for genome editing ¹⁵⁴. Oligos containing a T7 promoter sequence, the target sequence, and an additional constant region were created and annealed according to previously described methods ^{35,132}. These templates were

transcribed *in vitro* using T7 RNA polymerase (Promega) to generate single-stranded guide RNAs (sgRNAs) for microinjection. A linearized plasmid encoding *cas9*¹³⁰ was also transcribed *in vitro* using the SP6 mMessage mMachine Kit (Ambion). 200ng each of the sgRNA and *cas9* mRNA were combined and 2-4ng of this mixture was injected into early 1-cell stage embryos.

Genotyping zebrafish lines

The zebrafish mutants *valentino*^{b337 93}, *hnf1ba*^{hi2169 121}, *hoxb1b*^{um197} and *hoxb1a*^{um191 64} were genotyped as previously described. The *greb1*^{sa17608}, *egfl6*^{sa21615}, *sall4*^{sa14110} and *cellf2*^{sa33469} lines were identified via TILLING¹³¹ and mutant alleles were ordered from ZIRC. Mutant alleles were genotyped by sequencing PCR products amplified from genomic DNA using primers 5'-TGTGAAAATTTCTTGCTGTGT-3' and 5'-CTGAAGGGCAGAATACGG-3' for *greb1*^{sa17608}, 5'-ATCACAGATCCTGGGACAGC-3' and 5'-AAAAGCATTGGATGCA- GCTC-3' for *egfl6*^{sa21615}, 5'-GGGCATGAGGAGAGTATGGA-3' and 5'-TCTTTCAG- CCCACTGTCACTC-3' for *sall4*^{sa14110}, and 5'-CTTTGTTGGCGACCATTGA-3' and 5'-AAAGCGACAAAAACAGATTTCG-3' for *cellf2*^{sa33469}. *gbx1* mutants (alleles um300 and um301) were genotyped by Hpy188III restriction digest of PCR products amplified from genomic DNA using primers 5'-TGTCTCATTCGTCATTACCGTC-3' and 5'-AAGTTTCCGTGAAATTGAGGAG-3'. *gas6* mutants (alleles um296, um297, um298 and um299) were genotyped by

XcmI restriction digest of PCR products amplified from genomic DNA using primers 5'-GCGAACACATTGAGCAAGAA-3' and 5'-CATCG-CTAATGCTTCATCCA-3'

Genotyping embryos post ISH and immunostaining

hoxb1a, *hnf1ba* and *valentino* homozygous mutants are not viable as adults. As a result, all embryos assayed in this study were collected from crosses of heterozygous parents. After ISH and immunostaining, embryos were thoroughly rinsed in 1xPBS solution and individually genotyped. Representative genotyping data for *hoxb1a* mutants are shown in Figure 2.1. Embryos lacking r5 *krox20* staining represent *valentino* and *hnf1ba* homozygous mutants. Mutants ordered from ZIRC were genotyped as described above.

mRNA injections

All mRNAs for microinjection were synthesized *in vitro* using the Sp6 mMessage mMachine Kit (Ambion). 100ng/ul each of GFP¹⁴⁹ and *hoxb1a*¹¹⁰ mRNA were combined and 1-2ng of this mixture was injected into early 1-cell stage embryos.

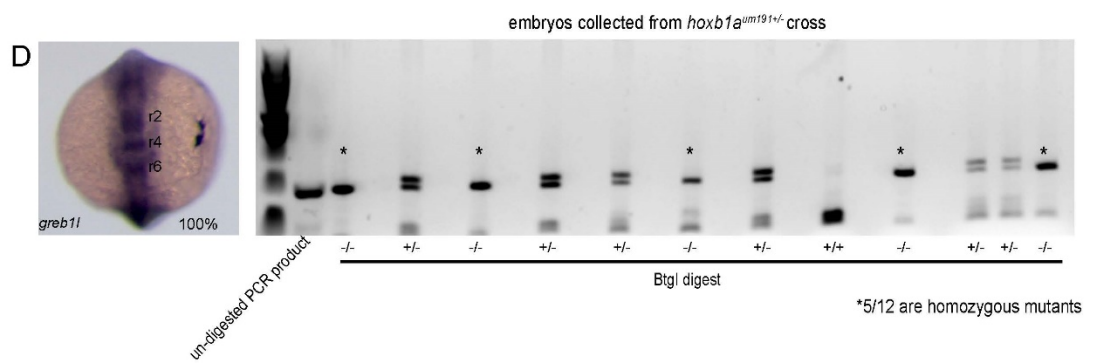
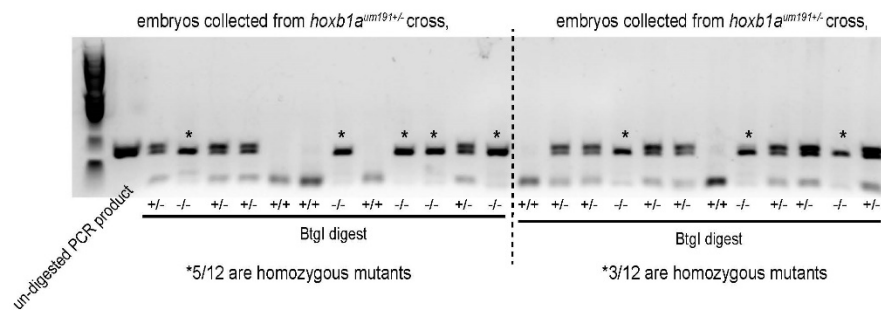
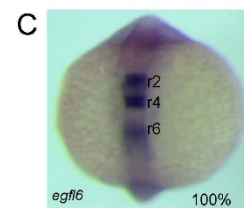
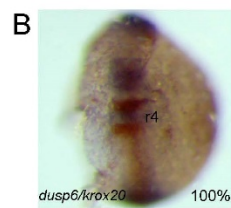
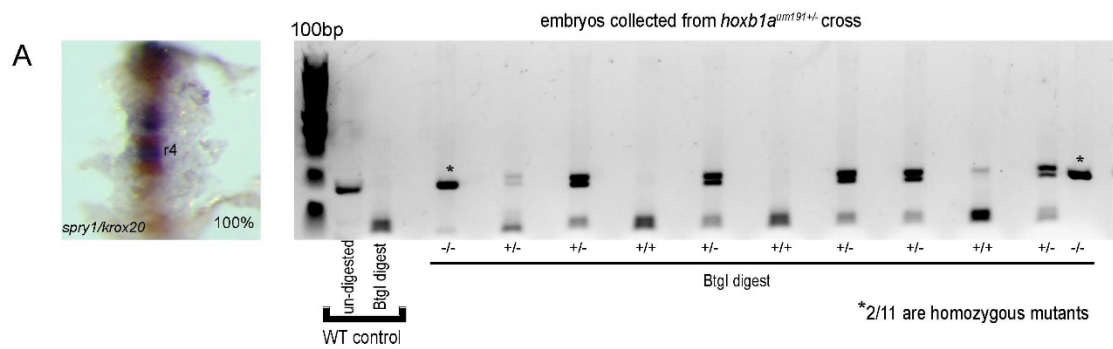


Figure 2.1 Genotyping of embryos collected from cross of *hoxb1a* heterozygous parents.

Several mutant lines used in this study are not viable as adults. As a result, many embryos used in assays were collected from crosses of heterozygous mutants. To ensure the presence of homozygous mutants in an assayed clutch, embryos were individually genotyped following ISH as outlined in the Methods section. Representative genotyping data for *hoxb1a* mutant embryos stained with (A) *spry1*, (B) *dusp6*, (C) *egfl6* and (D) *greb1l* demonstrate that approximately one quarter of the embryos assayed are homozygous mutant (indicated with asterisks), while 100% of the clutch showed normal staining for the assayed gene.

***Olig2* reporter line in *gas6* mutant background**

The transgenic line *Tg(olig2:EGFP)^{vu12 155}* was crossed into the *gas6* mutant background and subsequently a *gas6* homozygous mutant line was generated carrying the *olig2:eGFP* transgene. This line was used in preparing the RNA-seq library as well as studying the status of Olig2+ oligodendrocyte progenitor cells in mutant background.

RNA-seq library preparation

gas6 mutant embryos carrying the *olig2:EGFP* transgene were raised to 48hpf. Using the GFP signal as a guide, hindbrains were dissected from homozygous *gas6* transgenic mutants. Hindbrains were also dissected from *Tg(olig2:EGFP)^{vu12}* embryos as control samples. Pools of dissected tissues were deyolked and total RNA was extracted using the RNeasy Mini Kit (Qiagen). Similarly, WT and *hoxb1b^{um197}* mutants were collected at 18hpf and total RNA was extracted from pools of dechorionated, deyolked, whole embryos. For each RNA-seq experiment, three libraries were synthesized from 3µg RNA for each WT and mutant sample using the TruSeq Stranded mRNA Library Prep Kit (Illumina). All libraries were analyzed for quality on a bioanalyzer prior to sequencing (Agilent 2100 BioAnalyzer).

Processing and analysis of RNA-seq data

Fastq files were analyzed as previously described³⁵ using the University of Massachusetts Medical School Dolphin web interface¹⁵⁶. Differentially-expressed (DE) genes were identified as those with a greater than 2-fold change in expression between the WT and mutant samples. RNA-seq data is available at GEO under accession number GSE113437.

Quantitative PCR

Total RNA was extracted from whole embryos (WT and *hoxb1b*^{-/-} at 18 hpf), or from dissected hindbrain tissue (*gas6*^{-/-} and WT at 48 hpf) using the RNeasy Mini Kit (Qiagen). Approximately 100ng of RNA was used to reverse transcribe cDNA using the High Capacity cDNA Reverse Transcription Kit (Applied Biosystems). The qPCR reaction was carried out using SYBR Green qPCR Master Mix (BioTool) on an Applied Biosystems 7300 PCR System.

CHAPTER III: RESULTS

Derivation of expanded gene-sets expressed in r4 and r5/6 of the zebrafish hindbrain

To generate a list of candidate genes for function in the formation of r4-r6 of the vertebrate hindbrain, we turned to the gene expression database hosted at ZFIN¹⁵⁷. We downloaded the “Expression data for wildtype fish” file and searched for genes whose annotation include the terms “hindbrain”, “rhombomere 4”, “rhombomere 5” or “rhombomere 6”. This produced a list of 1,820 entries. We eliminated 146 records representing expressed sequence tags (ESTs), as these entries are not fully annotated, resulting in 1,674 genes. To further characterize these genes, we next manually reviewed the expression patterns deposited in ZFIN. Since we were particularly interested in genes controlling rhombomere formation, we excluded 480 genes that are only expressed later than 24hpf – when rhombomere formation is already completed – leaving 1,194 genes. We also expect genes controlling rhombomere formation to be expressed throughout the corresponding rhombomere. For instance, *hoxb1a* and *valentino*, which are respectively active in r4 and r5/r6 formation^{2,85,93}, are expressed throughout the entire corresponding rhombomere (referred to as “rhombomere-restricted”), while *islet1*, which is required for the differentiation of specific neurons, is expressed only in a subset of cells in each rhombomere. After restricting ourselves to genes expressed throughout one, or more, rhombomeres, we were left with 107 genes expressed in r4, r5 or r6 prior to 24hpf (Figure 3.1).

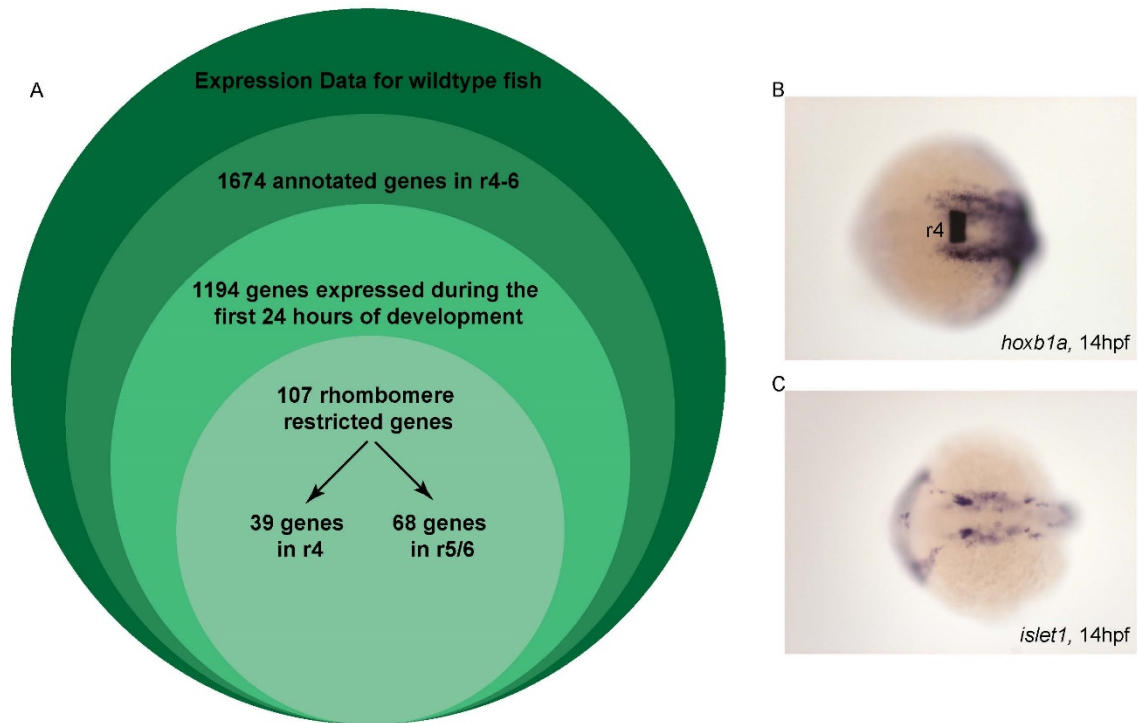


Figure 3.1 Identifying genes with expressions restricted to r4, r5 and r6.

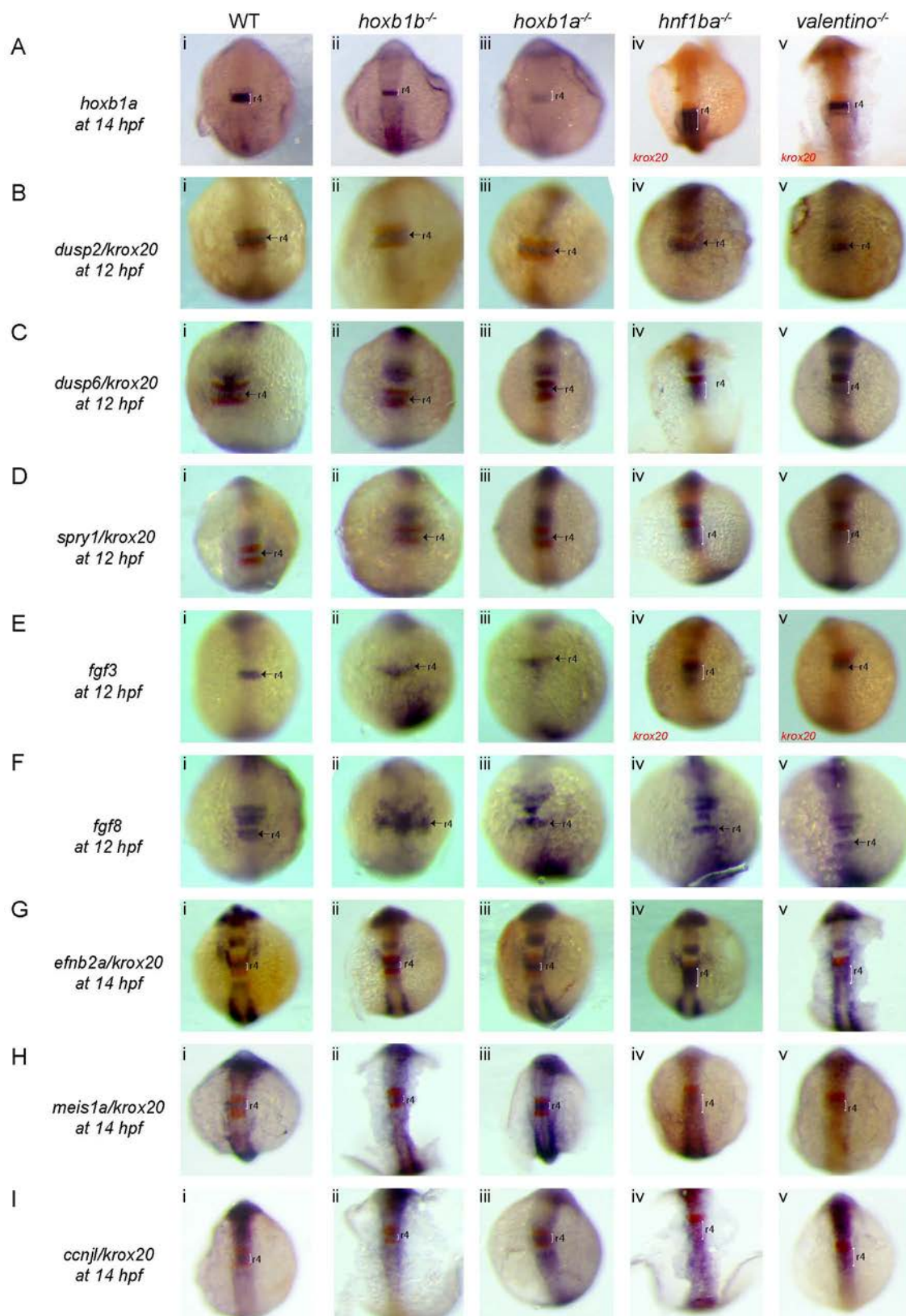
Panel (A) depicts the strategy employed to identify genes with expression patterns restricted to r4, r5 and r6 from the ZFIN data base. (B) Example of a “rhombomere-restricted” gene expression-*hoxb1a* expression is found throughout r4. (C) *islet1* marks only a few cells in the hindbrain and thus is not a “rhombomere-restricted” gene.

Specifically, 68 of these genes are expressed in r5 and/or r6 (r5/r6 gene-set), while 39 are expressed in r4 (r4 gene-set) (Appendix A and B). Notably, expression of these genes is not necessarily exclusive to r4 or r5/r6, but many of them are also expressed in additional rhombomeres – particularly r3. Our literature review revealed that a relatively small fraction of these 107 genes has previously reported roles in hindbrain formation. Specifically, eleven members of the r4 gene-set (28%) and twelve of the r5/r6 gene-set (18%) have been previously implicated in hindbrain formation (appendix A and B), indicating that a large number of uncharacterized genes are expressed in zebrafish r4-r6.

PG1 *hox* function is not required for expression of most r4 genes

We next set out to position the r4 and r5/r6 gene-sets within the GRN controlling caudal hindbrain formation. Previous work demonstrated that mutations in the PG1 *hox* TFs *hoxb1a* and *hoxb1b* disrupt proper hindbrain formation in zebrafish. In particular, *hoxb1b* mutants possess smaller r4 and r6, while *hoxb1a* mutants display a mis-specified r4^{62,64,124}, suggesting that PG1 *hox* TFs may regulate the r4 GRN.

To directly test if genes from the r4 gene-set are part of a PG1 *hox*-regulated r4 GRN, we analyzed expression of the r4 gene-set by ISH in *hoxb1a* and *hoxb1b* mutant zebrafish (Figure 3.2). Zebrafish *hoxb1b* is required for expansion of the r4 domain, but not for r4 formation^{62,64}. Accordingly, homozygous *hoxb1b*^{um197/um197} mutants (hereafter referred to as *hoxb1b*



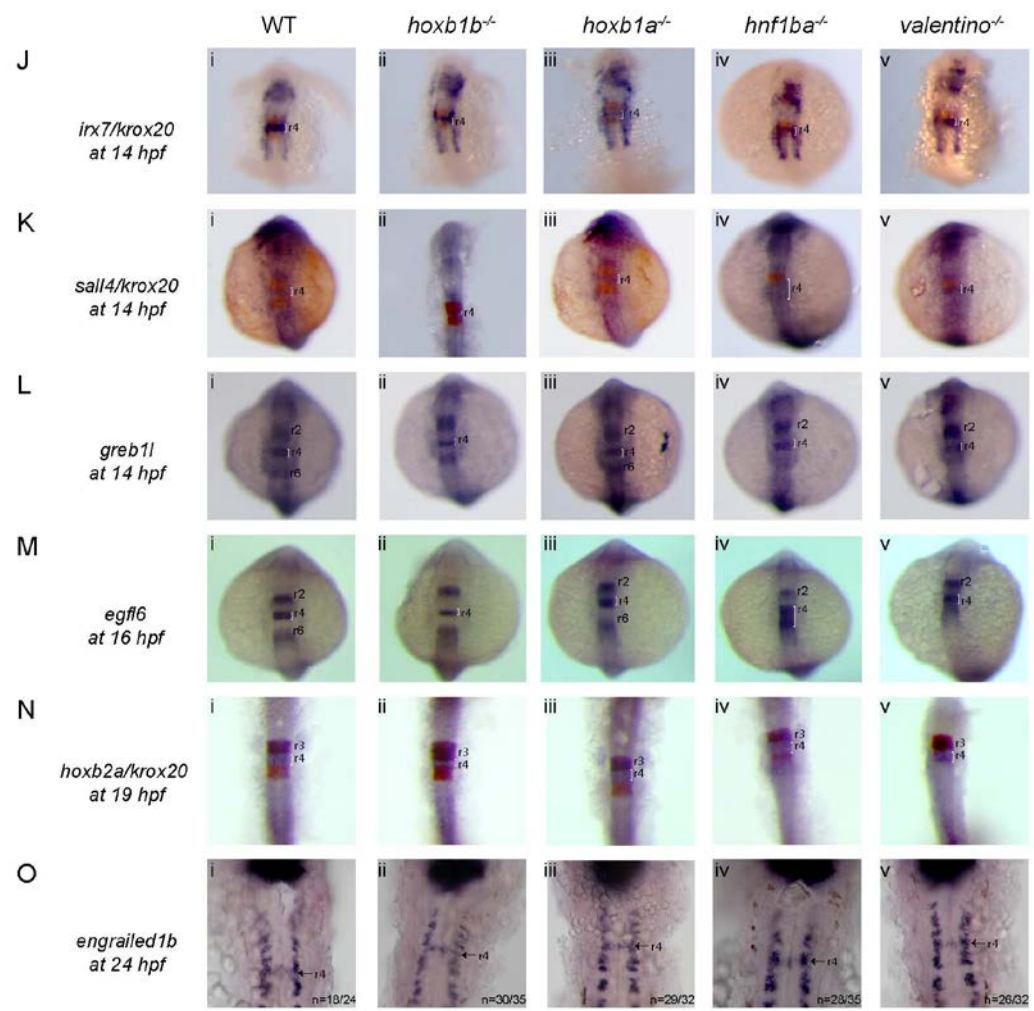


Figure 3.2 PG1 *hox* function is not required for expression of most r4 genes.

Expression of r4 genes was assayed via ISH in (i) WT, (ii) *hoxb1b* mutant, (iii) *hoxb1a* mutant, (iv) *hnf1ba* mutant and (v) *valentino* mutant zebrafish. The genes assayed include (A) *hoxb1a*, (B) *dusp2*, (C) *dusp6*, (D) *spry1*, (E) *fgf3*, (F) *fgf8*, (G) *efnb2a*, (H) *meis1a*, (I) *ccnjl*, (J) *irx7*, (K) *sall4*, (L) *greb1l*, (M) *egfl6*, (N) *hoxb2a* and (O) *engrailed1b*. *krox20* (red) which is expressed in r3 and r5, was used to assign the expression domains of several genes, as indicated. All embryos are oriented in dorsal view with anterior to the top. Embryos collected at 12hpf, 14hpf, 16hpf and 19hpf were imaged as whole-mounts. 24hpf embryos were flat-mounted for imaging. Black arrows point to the r4 domain in 12hpf and 24hpf embryos. White brackets mark the normal and expanded r4 domains in embryos staged at 14hpf, 16hpf and 19 hpf.

mutants) possess a narrow r4 domain that nevertheless expresses *hoxb1a* and is capable of generating both Mauthner cells and nVII facial neurons^{62,64}, albeit at a lower rate than wildtype r4. We generated ISH probes for 14 genes from the r4 gene-set and find that all 14 remain expressed in *hoxb1b* mutant fish, although their expression domains are reduced in size due to the smaller r4 (Figure 3.2, column ii). In contrast to *hoxb1b*, *hoxb1a* is required for r4 formation⁶⁴. In particular, homozygous *hoxb1a*^{um191/um191} mutant embryos (hereafter referred to as *hoxb1a* mutants), have reduced *hoxb1a* expression, lack r4-specific Mauthner cells, and the nVII facial neurons fail to migrate out of r4. While this disruption of r4 formation suggests that r4 gene expression might be generally reduced in *hoxb1a* mutants, we instead find that expression of the r4 gene set is unaffected also in *hoxb1a* mutants (Figure 3.2, column iii) and this expression persists at least until 24hpf (Figure 3.3).

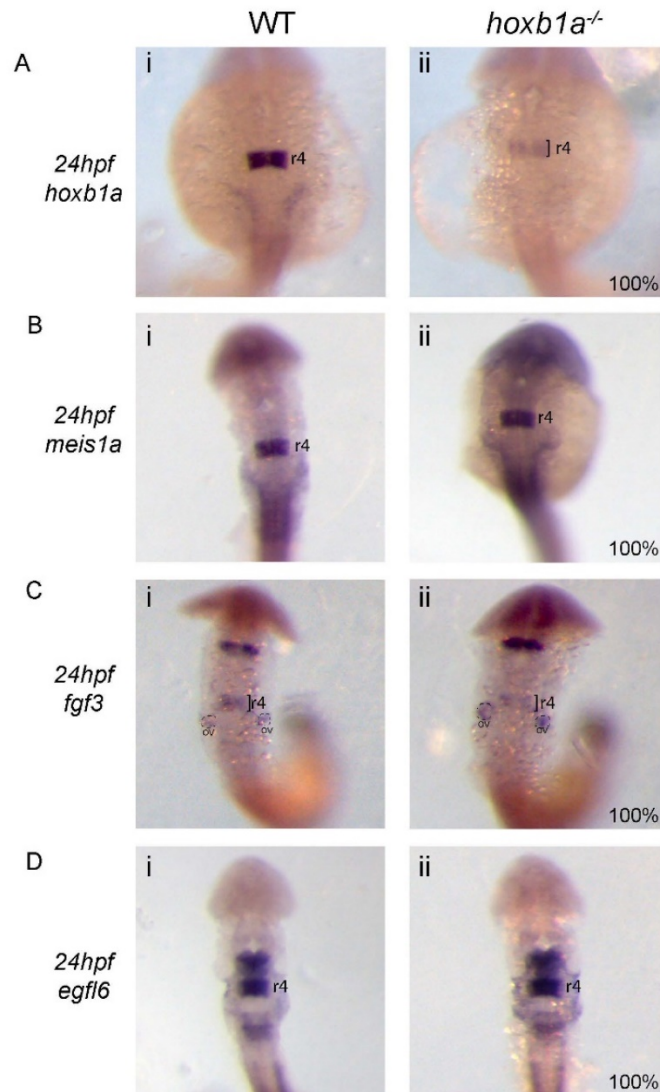


Figure 3.3 Expression of the r4 gene set is unaffected in *hoxb1a* mutants at least until 24 hpf.

Expression of *hoxb1a* (A), *meis1a* (B), *fgf3* (C) and *egfl6* (D) was assessed in wildtype (i) and *hoxb1a* mutant (ii) zebrafish at 24 hpf. The black brackets mark r4 and dotted circles represent the otic vesicles (OV).

Our finding that PG1 *hox* function is not required for expression of the 14 tested r4 genes led us to take a more broader approach and ask, which genes in the zebrafish are *hox*-dependent? To address this, we took advantage of the viability of homozygous *hoxb1b* mutants and used RNA-seq to identify *hoxb1b*-dependent genes during zebrafish embryogenesis. Comparing the *hoxb1b* mutant transcriptome to that of wildtype embryos revealed 866 differentially expressed genes at 18hpf (Figure 3.4). Comparison to the 1674 r4-6 genes identified in our database search (Figure 3.1A) demonstrate that only 85 hindbrain genes seem to be affected by the loss of *hoxb1b* (Appendix C). Thus, by this measure, only ~5% (85/1,674) of zebrafish caudal hindbrain genes are *hoxb1b*-dependent. Furthermore, of those 85 genes, only four (*mpz*, *fgf8a*, *cyp26b1* and *desma*) have rhombomere-restricted expression patterns (Appendix C). Also, while *fgf8* and *mpz* were identified as differentially expressed (up-regulated; Figure 3.4C) in *hoxb1b* mutants by our RNA-seq analysis, we did not detect changes in expression of these genes by ISH (Figures 3.2 Fii and 3.8 Cii) in the hindbrain.

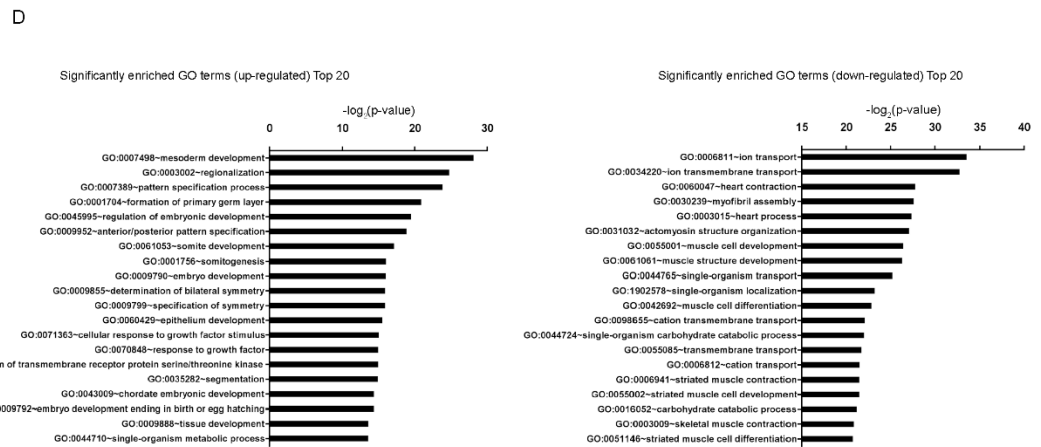
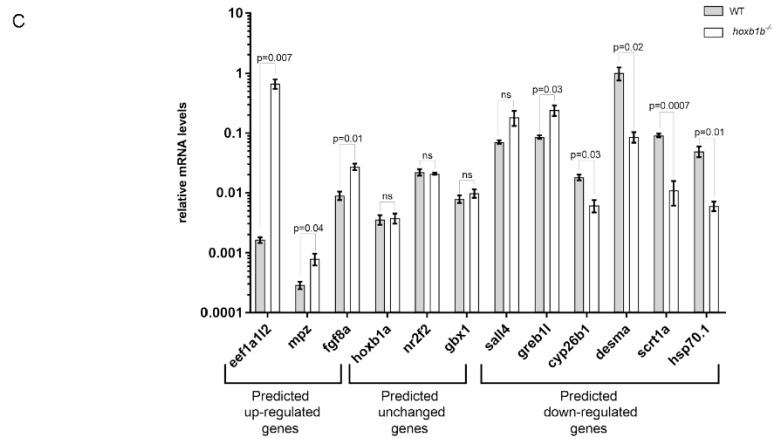
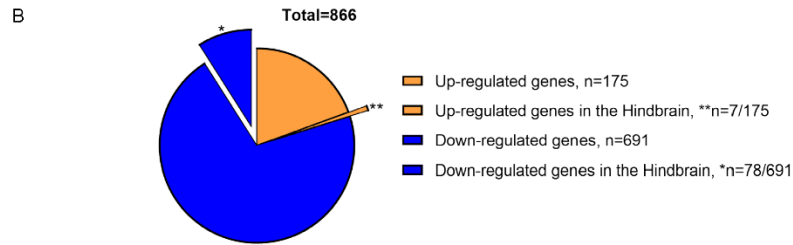
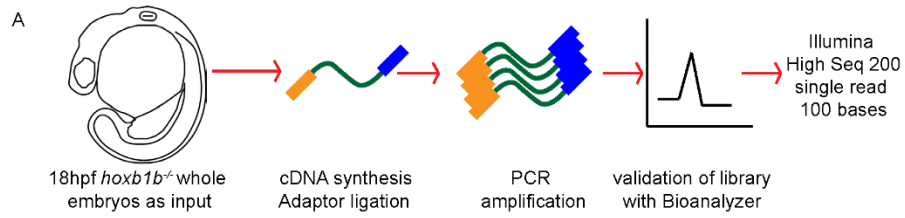


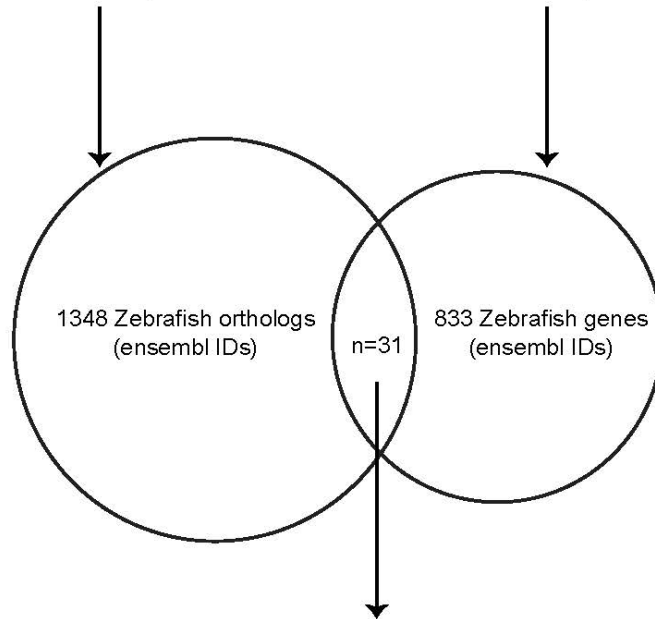
Figure 3.4 Generation and analysis of RNA-seq data from 18 hpf WT and *hoxb1b* mutant embryos. (A) Total RNA was collected from WT and *hoxb1b* mutant whole embryos and used for RNA-seq. (B) 866 differentially expressed genes were identified from RNA-Seq where seven of the 175 up-regulated genes and 78 of the 691 down-regulated genes are expressed in the hindbrain. (C) A subset of genes was validated by RT-qPCR from independently collected samples. (D) Top 20 GO terms for up-regulated and down-regulated genes.

Since RT-qPCR on independently collected samples validated our RNA-seq analysis (Figure 3.4C), it is possible that gene expression changes identified by RNA-seq are too subtle for detection by ISH. Indeed, the change in expression of *fgf8* and *mpz* is less than 2.5-fold and we find that the majority of *hoxb1b*-regulated genes identified by RNA-seq show relatively subtle changes in expression, such that ~93% of the down-regulated genes are reduced by less than 4-fold and only three genes are down-regulated by more than 10-fold (GEO under accession number GSE113437). Furthermore, GO-term analysis on differentially expressed genes show that *hoxb1b* is indeed involved in several important developmental processes (i.e. pattern specification, somatogenesis, segmentation, muscle development—to name a few) (Figure 3.4D).

We conclude that, while a large number of genes are *hoxb1b*-regulated in the zebrafish embryo, only a few of these genes are expressed in the caudal hindbrain and the observed changes in expression levels are relatively subtle. Hence, expression of most genes in r4 of the developing hindbrain do not require PG1 *hox* function. Further, a recent RNA-seq analysis of *Hoxa1* mutant mouse embryos (murine *Hoxa1* is functionally analogous to zebrafish *hoxb1b*) identified 1,537 *Hoxa1*-dependent genes¹⁴⁶, but only 31 genes are shared between the zebrafish and mouse data sets (Figure 3.5), suggesting that PG1 *hox* genes may in fact regulate distinct sets of genes in different species.

A. 1537 DE genes in *Hoxa1* mutant mice at 10.5dpc. De Kumar et.al., 2017

B. 866 DE genes in *hoxb1b* mutant zebrafish at 18hpf



C

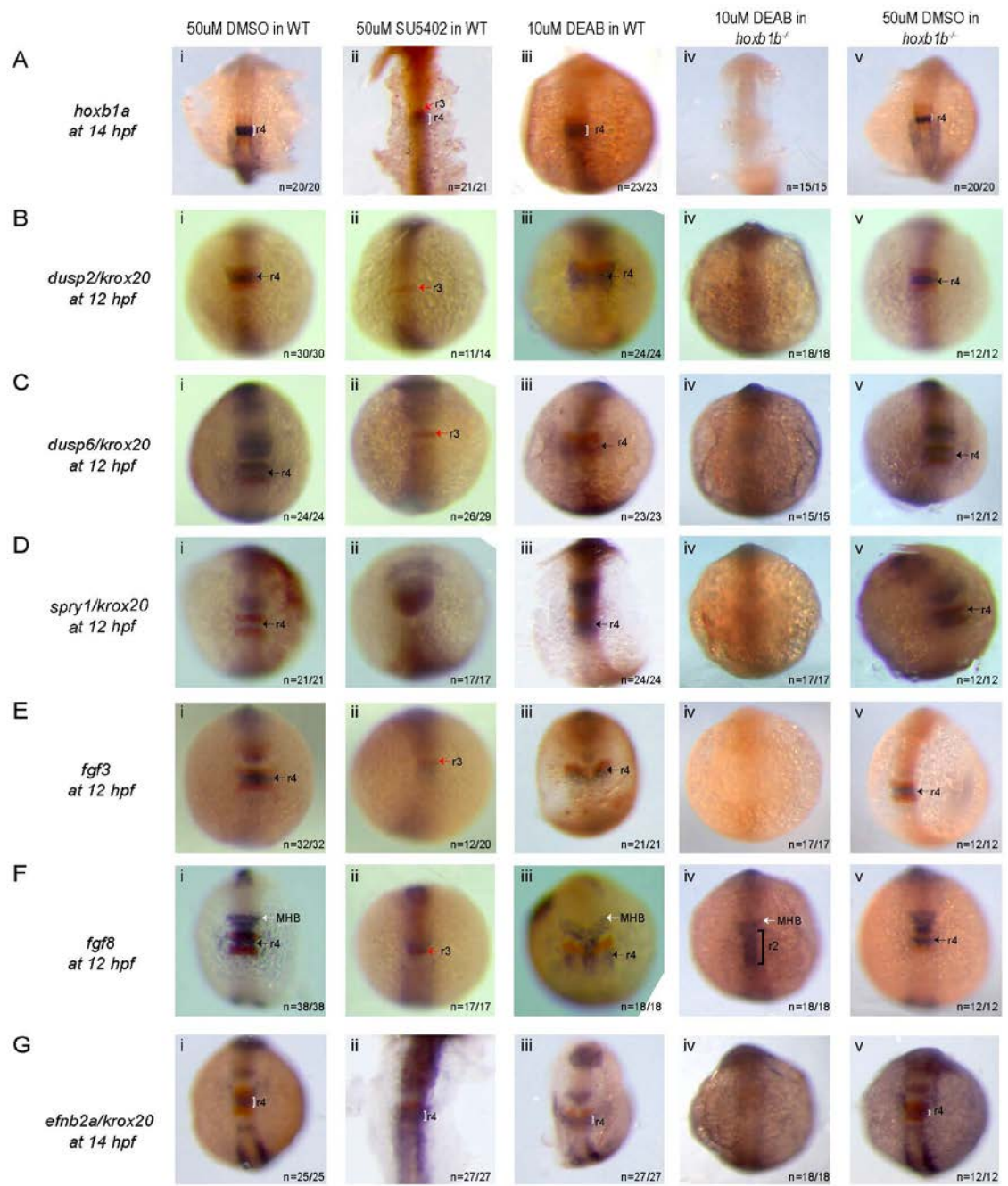
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coronin, actin binding protein, 1A(coro1a)
B-cell CLL/lymphoma 11Aa(bcl11aa)
integrin beta 3a(itgb3a)
collagen, type I, alpha 1b(col1a1b)
follistatin b(fstb)
troponin C type 1b (slow)(tnnc1b)
xenotropic and polytropic retrovirus receptor 1a(xpr1a)
Rh blood group, D antigen(rhd)
glutamate receptor, ionotropic, AMPA 2b(gria2b)
SH3 domain binding glutamate-rich protein(sh3bgr)
glypican 1b(gpc1b)
aldolase a, fructose-bisphosphate, b(aldoab)
internexin neuronal intermediate filament protein, alpha b(inab)
insulin induced gene 1(insig1)
solute carrier family 25 (mitochondrial carrier; phosphate carrier), member 3a(slc25a3a)
collagen, type I, alpha 2(col1a2)
zgc:153405(zgc:153405)
calsequestrin 1a(casq1a)
fibrinogen alpha chain(fga)
myosin, light polypeptide 3, skeletal muscle(myhz3)
immunoglobulin superfamily containing leucine-rich repeat 2(islr2)
neurofilament, medium polypeptide a(nefma)
ceruloplasmin(cp)
prolactin(prl)
monoacylglycerol O-acyltransferase 2(mogat2)
stathmin-like 4, like(stmn4l)
premelanosome protein a(pmela)
NOC3-like DNA replication regulator(noc3l)
histone 1, H4, like(hist1h4l)

Figure 3.5 Comparison between RNA-seq analyses of *Hoxa1* mutant mouse embryos and *hoxb1b* mutant zebrafish embryos.

RNA-seq analysis of *Hoxa1* mutant mouse embryos was recently published in ¹⁴⁶. Comparing the mouse data set (A) with the 866 differentially expressed genes identified by our RNA-seq (B) revealed an overlap of 31 genes (C). Notably, none of these 31 genes has a rhombomere restricted expression pattern.

A subset of r4 genes is regulated by FGF, but not RA, signaling

The fact that *hoxb1b* is not required for candidate r4 gene expression, suggests that other factors may be involved. In particular, the RA and FGF signaling pathways are known to function in hindbrain development ^{22,23,30–32,40}. To determine if expression of the r4 gene set is dependent on RA or FGF signaling, we treated wildtype embryos with 50uM SU5402 (a competitive inhibitor of the FGF receptor tyrosine kinase; ^{21,32}) or 10uM DEAB (a competitive inhibitor of RALDH, the enzyme required for conversion of retinaldehyde to retinoic acid; ^{40,64}). We find that inhibition of FGF signaling blocks expression of *dusp2* (Figure 3.6 Bii), *dusp6* (Figure 3.6 Cii), *spry1*(Figure 3.6 Dii), *fgf3* (Figure 3.6 Eii), and *fgf8* (Figure 3.6 Fii), in r4, but does not affect expression of *hoxb1a* or the remaining seven members of the r4 gene-set (Figure 3.6). Notably, all genes affected by loss of FGF signaling are themselves involved in the FGF signaling pathway, confirming the extensive use of feedback loops in this pathway ^{158–160}. In contrast, blocking RA signaling does not block expression of any of the r4 genes tested (Figure 3.6, column iii). The fact that expression of most r4 genes is not lost upon disrupting Hox TFs, FGF signaling or RA signaling suggests that they are either regulated independently of these signaling pathways or are under combinatorial control.



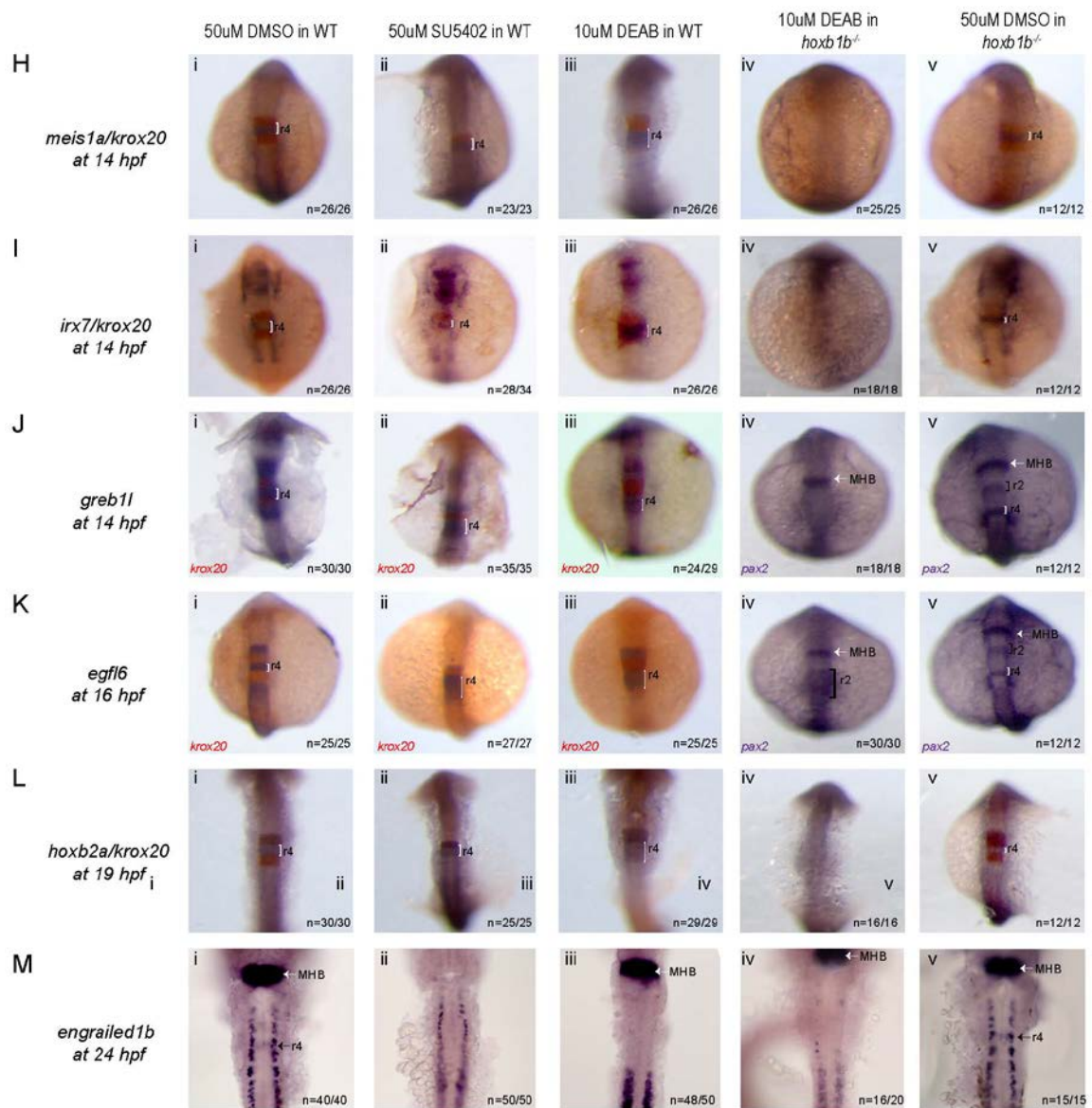


Figure 3.6 Simultaneous loss of *hoxb1b* and RA function disrupts

expression of r4 genes. Expression of r4 genes was assayed via ISH in (i) WT embryos treated with 50uM DMSO, (ii) WT embryos treated with 50uM SU5402 (iii) WT embryos treated with 10uM DEAB, (iv) *hoxb1b* mutant embryos treated with 10uM DEAB and (v) *hoxb1b* mutant embryos treated with 10uM DMSO. The genes assayed include (A) *hoxb1a*, (B) *dusp2*, (C) *dusp6*, (D) *spry1*, (E) *fgf3*, (F) *fgf8*, (G) *efnb2a*, (H) *meis1a*, (I) *irx7*, (J) *greb1l*, (K) *egfl6*, (L) *hoxb2a* and (M) *engrailed1b*. *krox20* (red), which is expressed in r3 and r5, was used to position the expression domains of several genes, as indicated. In panels (Jiv), (Jv), (Kiv) and (Kv), *pax2* is the second blue marker which labels the MHB. Black arrows point to r4, red arrows to r3 and white arrows to the MHB. White and black brackets indicate r4 and r2 size, respectively. All embryos are oriented in dorsal view with anterior to the top. Embryos collected at 12hpf, 14hpf, 16hpf and 19 hpf were imaged as whole-mounts. 24hpf embryos were flat-mounted for imaging. This figure also shows that a subset of r4 genes is regulated by FGF signaling (Bii, Cii, Dii, Eii and Fii) and that r4 genes are not affected by the loss of RA signaling (embryos in row iii).

Simultaneous loss of *hoxb1b* and RA function disrupts expression of r4 genes

We previously found that *hoxb1a* expression is unaffected when RA and *hoxb1b* function is disrupted independently, but is lost when these signals are disrupted simultaneously⁶⁴. To determine if the r4 gene-set is similarly regulated, we treated *hoxb1b* mutant embryos with 10uM DEAB and assayed gene expression by ISH. We find that expression of ten members of the r4 gene-set is completely lost when *hoxb1b* and RA signaling are simultaneously disrupted (Figure 3.6, column iv). Two genes, *fgf8* (Figure 3.6 Fiv), and *egfl6* (Figure 3.6 Kiv), show residual expression in the hindbrain, but these two genes are normally expressed also in the anterior hindbrain and previously published mouse data showed an expansion of the r2/r3 domains upon disruption of RA signaling¹³. Hence, the residual *fgf8* and *egfl6* expression detected in DEAB-treated *hoxb1b* mutants may be derived from r2/r3, not from r4. However, expression of *greb1l* (Figure 3.6 Jiv), (which is also expressed in the anterior hindbrain) is completely lost, indicating that not all genes are regulated in the same manner. A recent study reported subtle changes in *fgf3/8a* expression patterns in *hoxb1b* mutants and suggested that *hoxb1b* may regulate FGF signaling⁶². In line with this observation, we show that expression of FGF pathway components (*fgf3*, *fgf8*, *dusp2*, *dusp6* and *spry1*) is lost upon simultaneous disruption of *hoxb1b* and RA function, indicating that FGF signaling is downstream of *hoxb1b* and RA activity in the hindbrain. Notably, simultaneous

loss of *hoxb1b* and RA function has no effect on *pax2* (MHB in Figures 3.6 Jiv and Kiv) and *fgf8* (MHB in Figure 3.6 Fiv) expression at the mid-hindbrain boundary, indicating that co-regulation is specific to the region where *hoxb1b* is expressed. We carried out an analogous experiment to test if *hoxb1b* and FGF also cooperate to control r4 gene expression but find that even brief treatment of *hoxb1b* mutants with SU5402 dramatically disrupts embryogenesis (Figure 3.7), precluding us from assaying hindbrain gene expression. We conclude that expression of the r4 gene-set (including FGF pathway components) requires both *hoxb1b* and RA function.

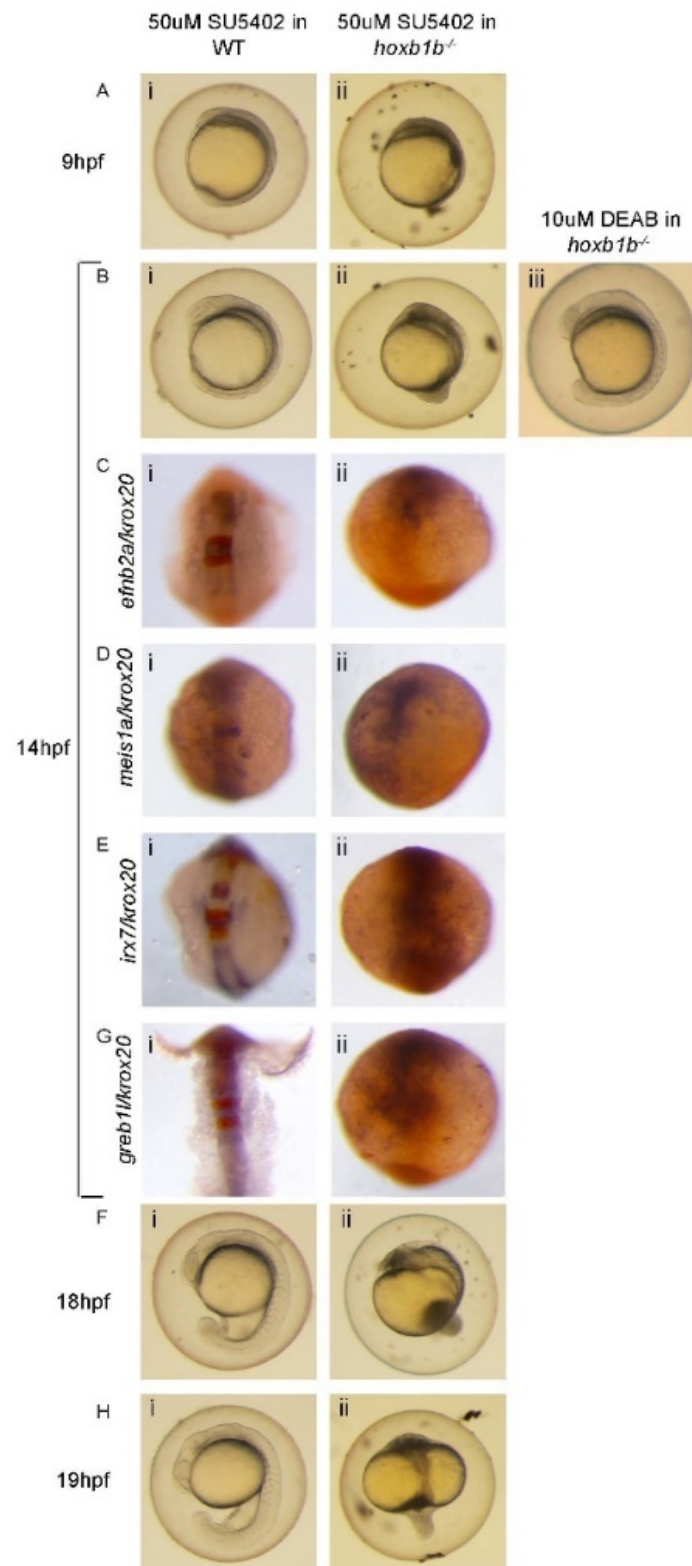


Figure 3.7 SU5402 disrupts embryogenesis in *hoxb1b* mutants.

Wildtype (i) and *hoxb1b* mutant (ii) zebrafish embryos were treated with SU5402 and assayed at various developmental stages by brightfield microscopy (A, B, F, H), or ISH to detect expression of *efnb2a/krox20* (C), *meis1a/krox20* (D), *irx7/krox20* (E) or *greb1l/krox20* (G). Note that defects in development are readily detectable in *hoxb1b* mutants treated with 50uM SU5402 (Aii, Bii), but not in WT embryos treated with SU5402 (Ai, Bi), nor in *hoxb1b* mutants treated with DEAB (Biii). As a result of these severe developmental defects, *hoxb1b* mutant embryos treated with SU5402 showed no specific staining for the r4 genes tested.

Expression of the r5/r6 gene-set is dependent on *hnf1ba* and *valentino* function

hnf1ba is the earliest-acting TF in zebrafish r5/r6 where it controls expression of the *mafB* gene *valentino*. Indeed, previous work demonstrated that both *hnf1ba* and *valentino* function is required for the expression of several r5/r6 genes⁸⁵. In order to determine if expression of our r5/r6 gene-set is also dependent on *hnf1ba* and *valentino*, we generated ISH probes for eight genes (*gbx1*, *tox3*, *sema3fb*, *mpz*, *gas6*, *cell2*, *nr2f2* and *col15a1b*) and assessed their expression in homozygous *hnf1ba*^{hi2169/hi2169} (referred to as *hnf1ba* mutant) and homozygous *valentino*^{b337/b337} (referred to as *valentino* mutant) embryos. For each of the eight genes, we find that expression is dramatically reduced in r5 and r6 of both mutant lines (Figure 3.8, columns iv and v).

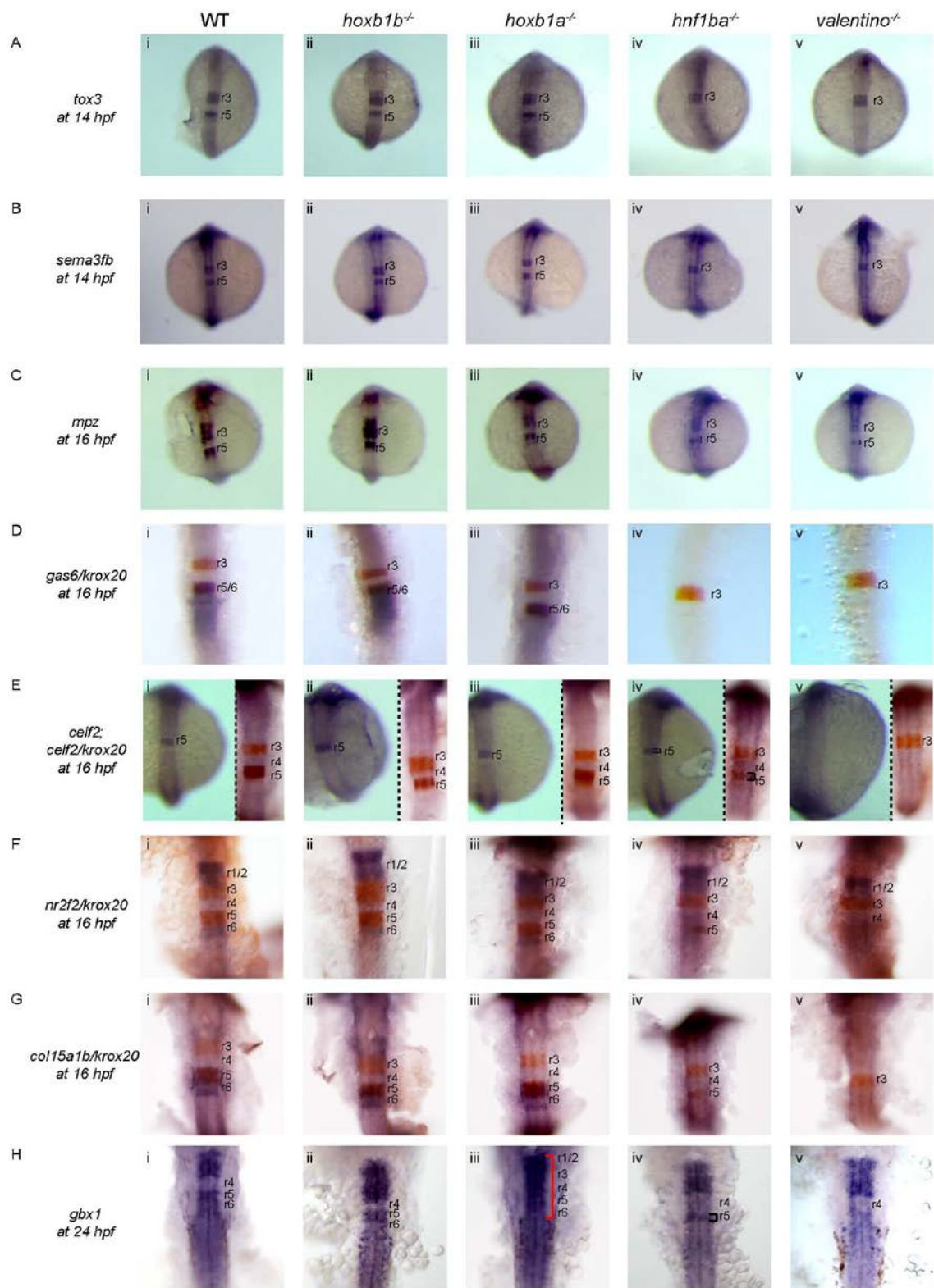


Figure 3.8 Expression of the r5/r6 gene-set is dependent on *hnf1ba* and

valentino. Expression of r5/r6 candidate genes was assayed via ISH in (i) WT, (ii) *hoxb1b* mutant, (iii) *hoxb1a* mutant, (iv) *hnf1ba* mutant and (v) *valentino* mutant zebrafish lines. The genes assayed include (A) *tox3*, (B) *sema3fb*, (C) *mpz*, (D) *gas6*, (E) *celf2*, (F) *nr2f2*, (G) *col15a1b* and (H) *gbx1*. *krox20* (red), which is expressed in r3 and r5, was used to position the expression domains of several genes as indicated. All embryos are oriented in dorsal view with anterior to the top. Embryos collected at 12hpf, 14hpf, 16hpf and 19hpf were imaged as whole-mounts. 24hpf embryos were flat-mounted for imaging. Black brackets mark the smaller r5 domain. Red bracket in (Hiii) indicates expression of *gbx1* throughout the hindbrain, as a result of reappearance of *gbx1* expression in r4 of *hoxb1a* mutants.

However, it is worth noting that the reduction in gene expression seen in *hnf1ba* and *valentino* mutants is somewhat variable. *tox3*, *sema3fb*, *gas6*, *nr2f2* and *col15a1b* appear to be completely lost in r5/r6 of both mutant lines, whereas residual expression of *cellf2* (Figure 3.8 Eiv) and *gbx1* (Figure 3.8 Hiv) is detected in *hnf1ba* mutants and weak *mpz* (Figure 3.8 Civ, v) expression is observed in both *hnf1ba* and *valentino* mutants. This is in agreement with previous reports⁹¹ demonstrating that slight expression of some r5 genes persists in these mutants.

hoxb1b is initially broadly expressed in the caudal hindbrain^{17,40} and previous work demonstrated that several r5/r6 genes (including seven genes from the r5/r6 gene set; *zwi*, *gas6*, *mpz*, *tox3*, *mmp2*, *nab1a*, and *nck2a*) are up-regulated following *hoxb1b* overexpression¹⁴⁹, suggesting that *hoxb1b* may regulate gene expression in r5/r6. We therefore assayed expression of the r5/r6 gene-set also in *hoxb1b* and *hoxb1a* mutant embryos (Figure 3.8, column ii and iii) but detect no changes in r5/r6 gene expression. We conclude that *hnf1ba* and *valentino*, but not *hoxb1b* or *hoxb1a*, are required for gene expression in r5 and r6.

Expression of the r5/r6 gene-set requires FGF and RA signaling

Previous work demonstrated that FGF signaling is required for r5/r6 formation^{21,32,33}. Since *hnf1ba* expression is independent of FGF^{32,33}, FGF must control r5/r6 formation downstream of *hnf1ba*. Indeed, FGF reportedly acts together with *hnf1ba* to regulate *valentino* and *krox20* expression in r5^{85,87}.

Hence, by determining if the r5/r6 gene-set is FGF independent or dependent, we can better understand the GRN controlling r5/r6 formation. Additionally, RA is required for formation of r5/r6 and for the expression of r5/r6-restricted genes such as *hnf1ba*, *valentino*, and *krox20*¹⁷. Strikingly, our analyses revealed that inhibition of either FGF or RA signaling in wildtype embryos blocks expression of all genes in the r5/r6 gene-set (Figure 3.9), with the one exception of a narrow domain of residual *nr2f2* expression in r6 (Figure 3.9 Fii) of SU5402 treated embryos. Hence, r5/r6 gene expression is dependent both on the activity of the *hnf1ba* and *valentino* TFs, as well as on RA and FGF signaling.

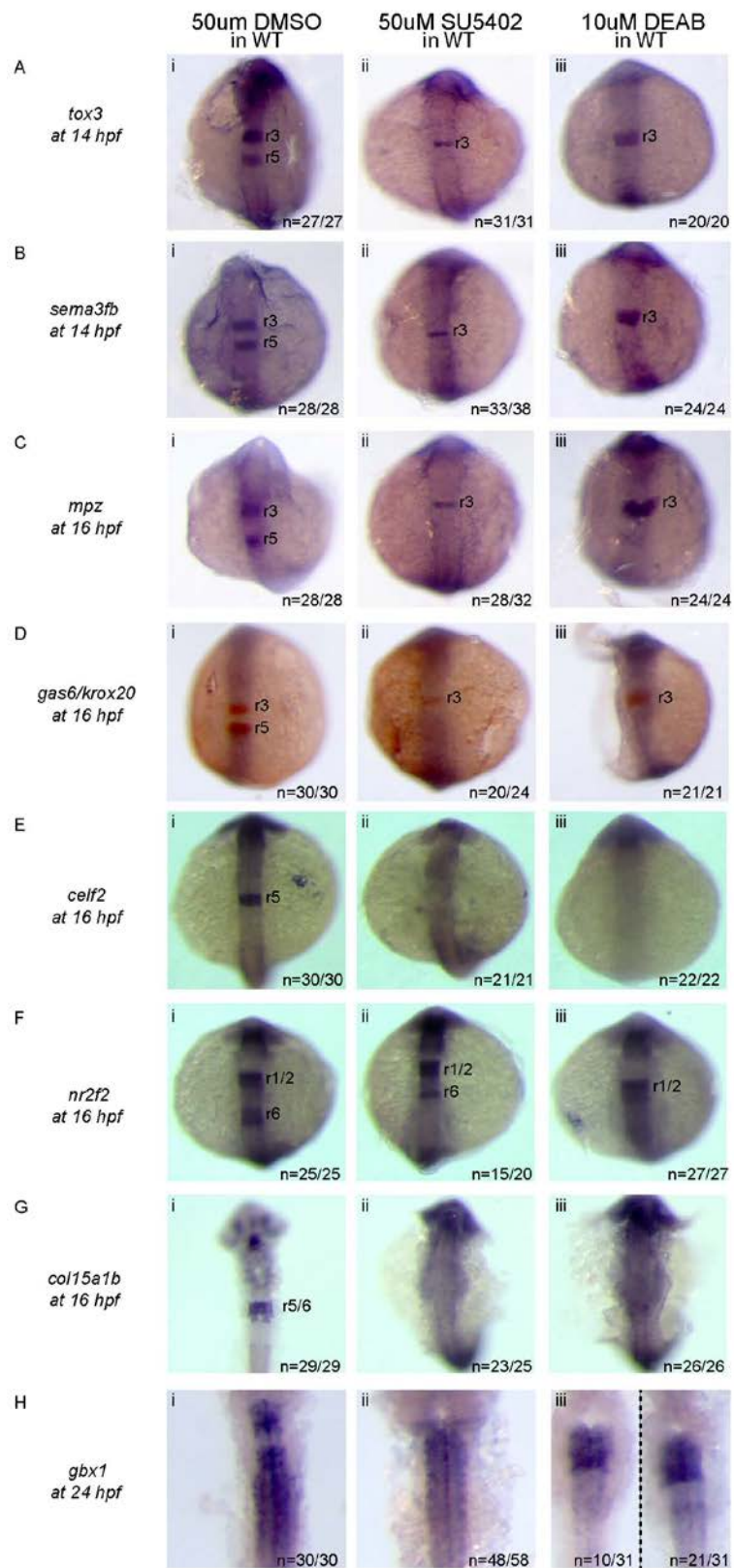


Figure 3.9 Expression of the r5/r6 gene-set requires FGF and RA signaling.

Expression of r5/6 genes was assayed via ISH in (i) WT embryos treated with 50uM DMSO, (ii) WT embryos treated with 50uM SU5402 *and* (iii) WT embryos treated with 10uM DEAB. The genes assayed include (A) *tox3*, (B) *sema3fb*, (C) *mpz*, (D) *gas6*, (E) *celf2*, (F) *nr2f2*, (G) *col15a1b* and (H) *gbx1*. *krox20* (red), which is expressed in r3 and r5, was used to position the expression domain of some genes as indicated. All embryos are oriented in dorsal view with anterior to the top. Embryos collected at 12hpf, 14hpf, 16hpf and 19hpf were imaged as whole-mounts. 24hpf embryos were flat-mounted for imaging.

***hnf1ba* establishes the posterior boundary of r4 gene expression**

Gene expression boundaries in the developing hindbrain are initially established via repressive interactions at the level of transcription. For instance, *hoxb1a*, *efnb2a* and *fgf3* expression expands caudally from r4 into presumptive r5 in *hnf1ba* mutants^{85,116}, indicating that *hnf1ba* represses r4 gene expression (directly or indirectly) to establish the r4/r5 border. In order to determine if the expression domains of genes in the r4 and r5/r6 gene-sets are similarly established by repressive interactions, we examined r4 gene expression in *hnf1ba* and *valentino* mutants, as well as r5/r6 gene expression in the PG1 *hox* mutants.

For the r4 gene-set, we find that *dusp6* (Figure 3.2 Civ), *spry1* (Figure 3.2 Div), and *egfl6* (Figure 3.2 Miv), show expansion of the r4 expression domain into r5 of *hnf1ba* mutants, while *fgf8*, *irx7*, *greb1l* and *eng1b* expression is not affected. In contrast, expression of the r4 gene-set is not affected in *valentino* mutants, with the exception of *efnb2a* (Figure 3.2 Gv), which may show a slight expansion into r5 (previously shown in⁸⁵). For the r5/r6 gene-set (with the exception of *gbx1*) we do not observe expansion into r4 in either of the PG1 *hox* mutants (Figure 3.8, columns ii and iii). We conclude that *hnf1ba* restricts expression of many, but not all, genes in the r4 gene-set to presumptive r4, but that *valentino* and the PG1 *hox* genes are not required to establish gene expression boundaries for the r4 and r5/r6 gene-sets.

***gbx1* expression requires *hnf1ba* and *valentino* in r5/r6 and is repressed by *hoxb1a* in r4**

The *gbx1* gene displays an interesting expression pattern in that it is expressed throughout the hindbrain at 24hpf, except in r4 (Figure 3.8 Hi). To better understand the regulation of *gbx1* expression, we analyzed its expression pattern in *hoxb1b*, *hoxb1a*, *hnf1ba* and *valentino* mutants. We find that *gbx1* expression is lost in r5/r6 of *valentino* mutants and that only a narrow expression domain persists in r5 of *hnf1ba* mutants (Figure 3.8 Hiv, v), indicating that *hnf1ba* and *valentino* are required for *gbx1* expression in r5/r6. Strikingly, *gbx1* expression is restored to the r4 domain of 24hpf *hoxb1a* mutant embryos, but not of *hoxb1b* mutant embryos (Figures 3.8 Hiii and 5Ai), suggesting that *gbx1* might be repressed by *hoxb1a* in r4. *gbx1* is actually expressed in wildtype r4 at ~10hpf, but this expression disappears coincident with the onset of *hoxb1a* expression (Figures 3.10), again suggesting that *hoxb1a* may repress *gbx1* expression. To test this directly, we overexpressed *hoxb1a* by injection of synthetic mRNA into 1-2 cell stage embryos and assayed *gbx1* expression at 24hpf by ISH. We find that ~80% (27/34) injected embryos display a clear decrease in *gbx1* hindbrain expression (Figure 3.10). We conclude that *hoxb1a*, either directly or indirectly, represses *gbx1* expression in r4 (Figures 3.10) and that its expression in r5/r6 is regulated similarly to the genes in the r5/r6 gene-set.

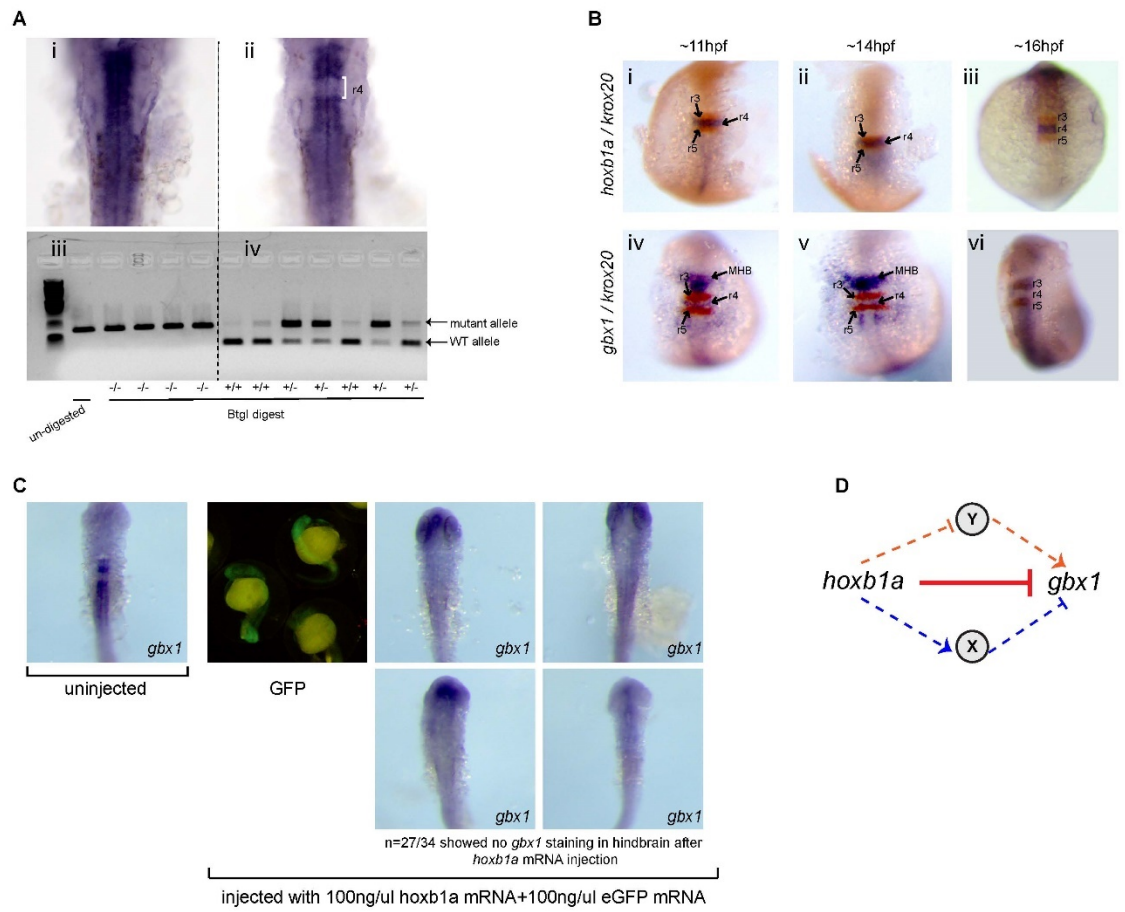


Figure 3.10 *gbx1* expression is repressed by *hoxb1a* in r4.

(A) *gbx1* expression is restored in *hoxb1a* mutant embryos. Embryos from a cross of *hoxb1a* heterozygous parents were assayed by ISH for *gbx1* expression, producing two phenotypes (i, ii). Subsequent genotyping revealed that homozygous *hoxb1a* mutants express *gbx1* in r4 (ii, iv). (B) *gbx1* is initially expressed in r4 (iv), but disappears (v, vi) when *hoxb1a* expression is activated (i, ii, iii). (C) A mixture of *hoxb1a* and GFP mRNA was injected into 1-cell stage embryos and successfully injected embryos (identified by GFP expression) were stained for *gbx1* expression at 24hpf. The observed reduction in *gbx1* expression demonstrates that *hoxb1a* is capable of repressing *gbx1* expression. (D) Hypothetical model depicting the potential relationship between *gbx1* and *hoxb1a*. *hoxb1a* could either repress *gbx1* directly (solid red T bar) or indirectly by activating a repressor (X; blue arrow) or repressing an activator (Y; orange T bar).

***gas6, gbx1, sall4, egfl6, celf2* and *greb1l* function is not required for r4-r6 formation**

Considering the number of genes assigned to the r4 and r5/r6 gene-sets by our database search, it is surprising that previous large-scale mutagenic screens identified only a few genes required for r4-r6 formation^{93,112,117}. While this finding may indicate redundancy in the r4 and r5/r6 GRNs, the fact that targeted mutagenesis studies have identified additional genes required for r4-r6 formation (e.g. PG1 *hox* genes^{56,60,62,64,161,162} and *krox20*^{108,163}) may instead suggest that the original screens may not have reached saturation. To directly test if genes in the r4 and r5/r6 gene-sets are required for rhombomere formation, we selected six genes (*gas6, sall4, egfl6, celf2, greb1l* and *gbx1*) for further analysis. Germline mutations for four of these genes (*sall4, egfl6, celf2* and *greb1l*) have been generated by community-based mutagenesis projects^{131,164} and are available from the zebrafish resource center (ZIRC; Table 3.1). These four mutations were procured from ZIRC in the form of fertilized embryos and raised in our laboratory. Genotyping and sequencing confirmed the presence of the expected mutations (Table 3.1) (Figure 3.11).

Table 3.1 Mutant lines obtained from ZIRC

Gene name	Mutant ID	Chromosome location	Exon affected	Mutation	Consequence	Amino acid affected
<i>grebl1</i>	sa17608	chr2:11980696	1 of 32	T>A	nonsense	41 of 1942aa
<i>celf2</i>	sa33469	chr4: 17566280	2 of 13	A>T	nonsense	28 of 514aa
<i>egfl6</i>	sa21615	chr9: 54710599	8 of 12	G>A	disrupted splice site	277 of 506aa
<i>sall4</i>	sa14110	chr23: 39233081	2 of 4	C>T	nonsense	695 of 1091aa

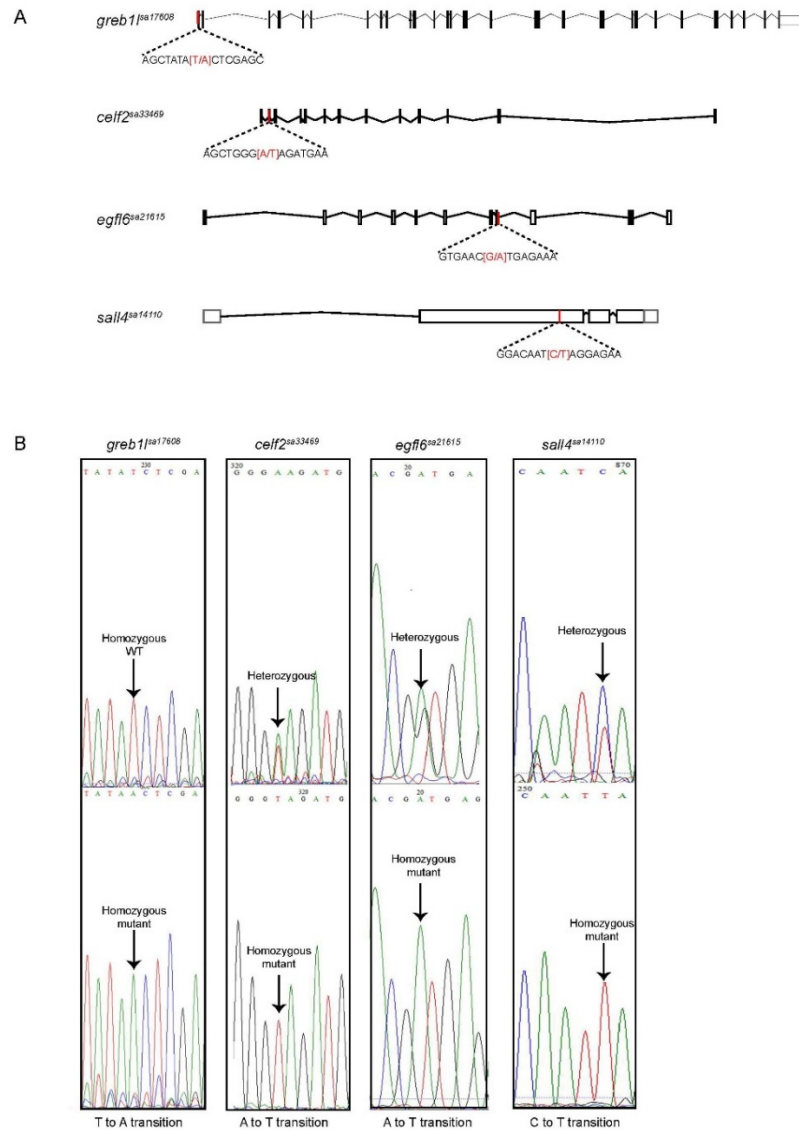


Figure 3.11 Genotyping data for *sall4*, *egfl6*, *celf2* and *greb1* mutants.

sall4, *egfl6*, *celf2* and *greb1* mutants generated by TILLING were procured from ZIRC. In each case, the mutation introduces a single nucleotide change (A; red text) causing a premature stop codon, except for *egfl6* where the point mutation disrupts an essential splice site in exon 8. (B) Sequencing traces showing expected single nucleotide changes in each mutant line.

Since *gas6* and *gbx1* mutants were not available from the resource center, we generated these by CRISPR/Cas9-mediated mutagenesis. We designed a sgRNA targeting the *gas6* start codon in exon 1 (Figure 3.12A) and confirmed its activity by microinjection into a pool of 1-cell stage embryos followed by screening for disruption of a diagnostic XcmI site at the genomic target site (Figure 3.12B). Embryos injected with the sgRNA were then raised to adulthood and founders identified by their ability to pass mutations to their offspring (Figure 3.12C). In this manner, we identified two *gas6* mutant founders from five fish screened. The founders were crossed to wildtype fish and the offspring raised as the F1 generation. One founder did not transmit mutation to its offspring (n=0/92), but the other transmitted mutations to 20% (n=12/60; Table 3.2) of its F1 offspring. Sequencing revealed that this founder transmitted four different mutant alleles (Appendix D) where each allele carried a different four nucleotide deletion, but translation of each mutant allele is nevertheless predicted to produce an out of frame product that terminates at the same premature stop codon (residue 99; Figure 3.12D) (Appendix D). ISH and RT-qPCR analyses further revealed that *gas6* transcripts are lacking in *gas6* mutants, possibly as a result of nonsense-mediated mRNA decay (Figure 3.12E).

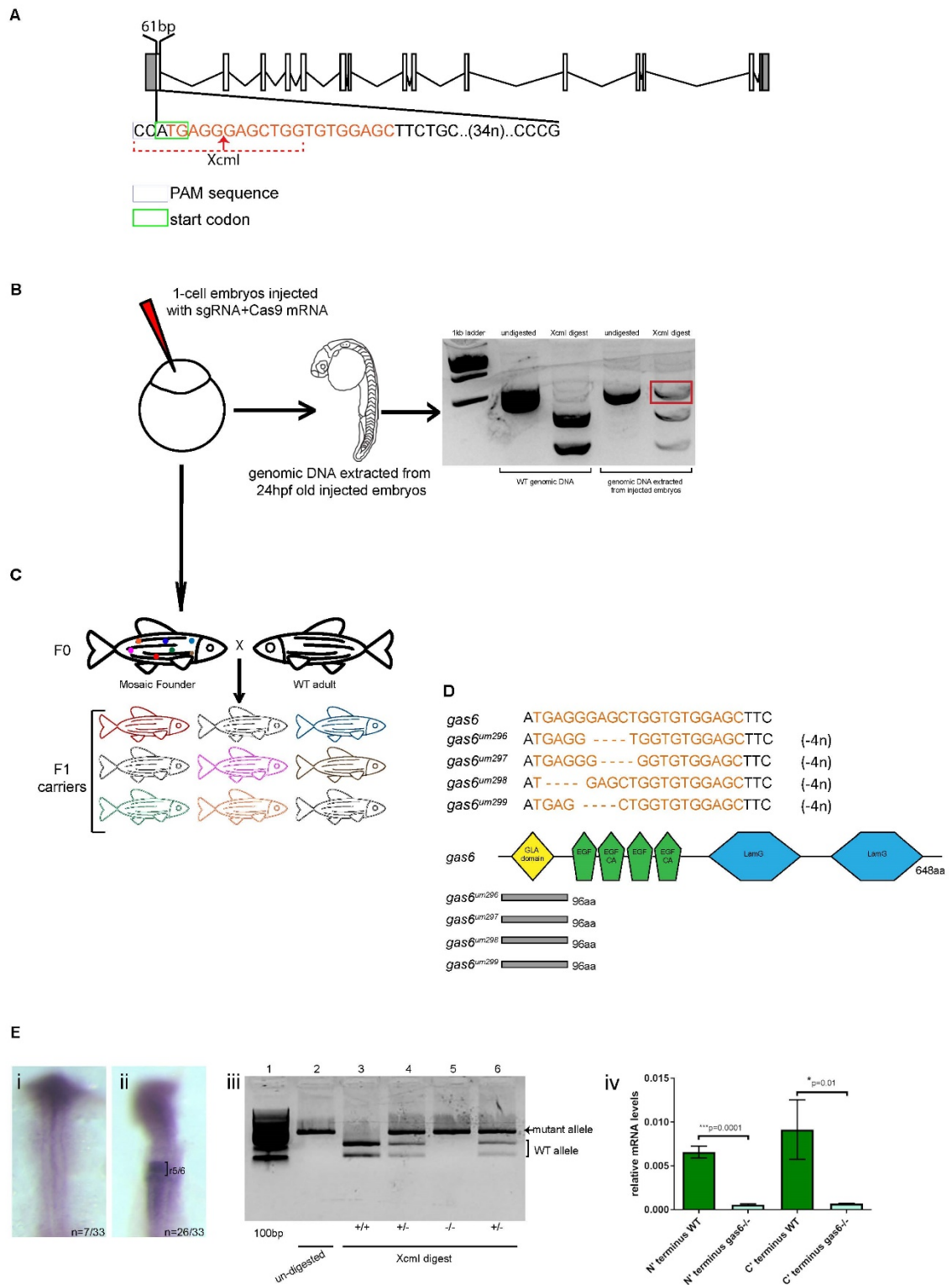


Figure 3.12 Scheme for generating *gas6* mutant line.

(A) Schematic showing the 20 nucleotide (orange text) target site in exon 1 of *gas6*. CAA represents the PAM sequence (blue box) and ATG (green box) is the start codon. XcmI target sequence is indicated by the dotted red line, the red arrow denotes the cut site. (B) sgRNA and Cas9 mRNA was injected into 1-cell stage embryos. Injected embryos were raised to 24hpf and genomic DNA was extracted from a pool of embryos. XcmI digest of PCR products amplified from genomic DNA reveal the presence of a mutation (red box in gel). (C) Injected embryos were raised to generate F0 adults which were crossed with WT adults to raise the F1 generation. At 3 months age, genomic DNA was extracted from fin-clips from individual F1 fish and genotyped as in panel B. (D) Sequencing of F1 genomic DNA revealed transmission of four different mutant alleles (um296, um297, um298, um299), each with a different 4 nucleotide deletions (orange dashes). Each mutant allele codes for 96 out of frame amino acids (gray boxes) followed by a premature stop codon. (E) One quarter of the embryos collected from a cross of two heterozygous parents lack *gas6* expression in r5/6 (i). XcmI digest of PCR products amplified from genomic DNA extracted from embryos lacking *gas6* expression were homozygous for mutant *gas6* allele (iii, lane 5). cDNA was synthesized from total RNA extracted from WT and homozygous *gas6* mutant fish. Quantitative RT-PCR using two different primer pairs (targeting the N and C termini, respectively) shows that homozygous *gas6* mutants have significantly lower levels of *gas6* mRNA (iv).

Table 3.2 Summary of transmission and viability of mutant lines

Gene name	F0 generation	F1 generation	F2 generation
<i>gas6</i>	5 fish screened, 2 founders	Founder 1: 12/60 carried mutations; transmitted 4 different alleles, each resulting in a frame-shift mutation Founder 2: 0/92 carried mutations	5/16 homozygous mutant
<i>gbx1</i>	2 fish screened, 2 founders,	Founder 1: 34/48 carried mutations; transmitted 4 different alleles – 2 resulted in frame-shift mutations, 2 did not Founder 2: did not produce offspring	0/15 homozygous mutant
<i>sall4</i>	N/A	*8/24	5/21 homozygous mutant
<i>greb1l</i>	N/A	*10/24	5/34 homozygous mutant
<i>celf2</i>	N/A	*12/24	**1/21 homozygous mutant
<i>egfl6</i>	N/A	*16/24	***6/24 homozygous mutant

*ZIRC sent us the offspring of an F1 heterozygous carrier and WT; thus, 50% should be heterozygous carriers

**1 homozygous fish identified, it was crossed with a heterozygous sibling for all in situ analyses

***homozygous mutants do not breed, all in situ analyses were thus done on crosses of heterozygous carriers

In an analogous fashion, a sgRNA was designed to a target site 288 nucleotides downstream of the START codon in exon 1 of *gbx1* (Figure 3.12A). Efficacy of the sgRNA was determined using a diagnostic Hpy188III site and we identified two founders which were used to raise the F1 generation (Figure 3.13B) (Table 3.2). Of the two founders, only one produced offspring and this founder transmitted two different mutations. Both of these alleles produce premature STOP codons N-terminal to the homeodomain (Figure 3.13D) (Appendix D).

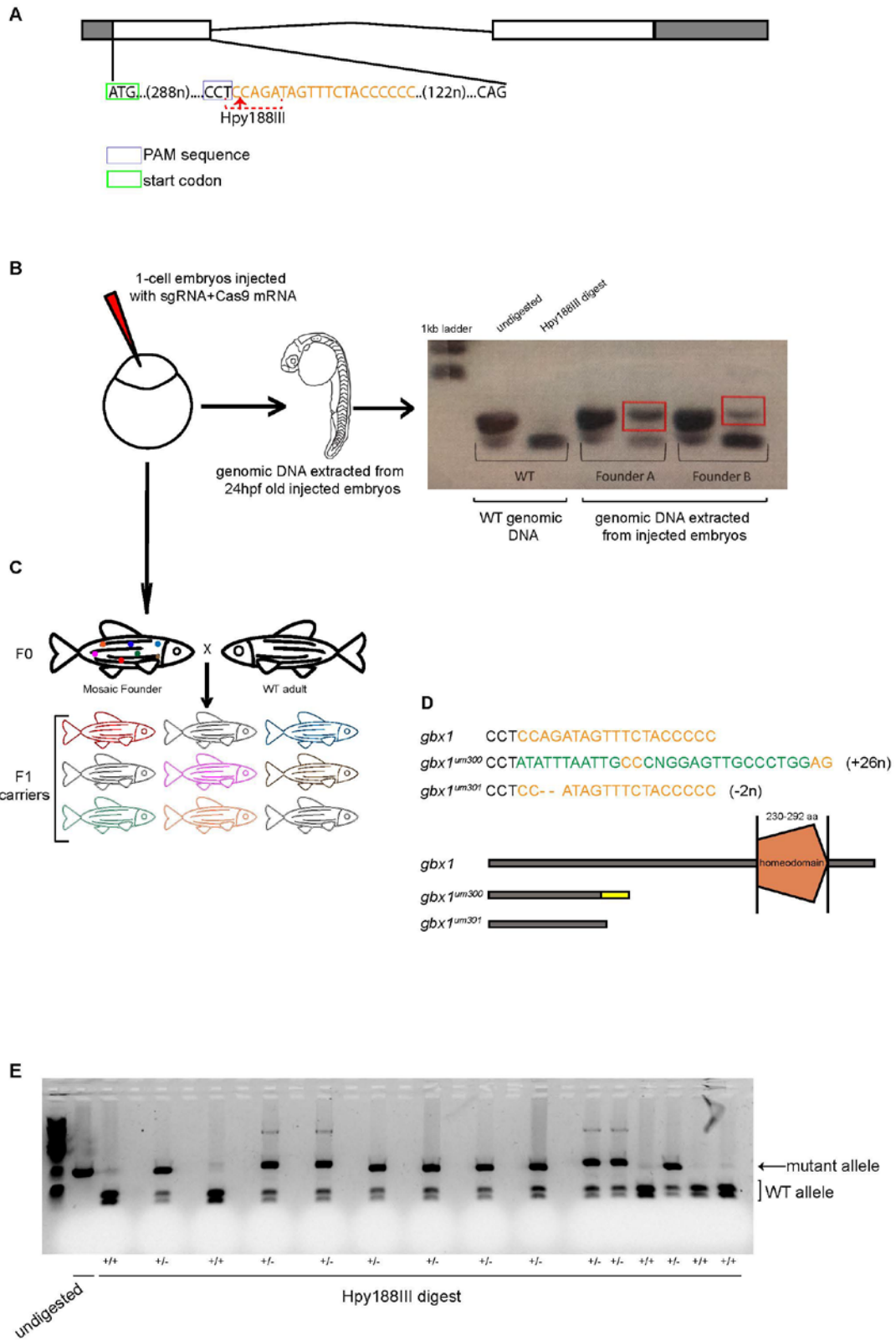


Figure 3.13 Scheme for generating *gbx1* mutant line.

(A) Schematic showing the 20 nucleotide (orange text) target site in exon 1 of *gbx1*. CCT represents the PAM sequence (blue box) and ATG (green box) is the start codon. Hpy188III target sequence is represented by the dotted red line, the red arrow denotes the cut site. (B) sgRNA and Cas9 mRNA was injected into 1-cell stage embryos. Injected embryos were raised to 24hpf and genomic DNA extracted from a pool of embryos. Hpy188III digest of PCR products amplified from genomic DNA (extracted from injected embryos) reveal the presence of a mutation (red boxes in gel). (C) Injected embryos were raised to give rise to F0 adults. These fish were crossed with WT adults to raise the F1 generation. At 3 months age, genomic DNA was extracted from fin-clips of individual F1 fish and genotyped as described in panel B. (D) Sequencing of F1 genomic DNA revealed transmission of two different mutant alleles; one allele (um300) has a 26-nucleotide insertion (green text) and the second allele (um301) has a two-nucleotide deletion (orange dashes). The resulting amino acid sequence is shown in the form of grey (amino acid sequence identical to wildtype) and yellow (out of frame amino acid sequence) boxes. Both mutant alleles result in premature stop codons upstream of the homeodomain. (E) Hpy188III digest of PCR products amplified from genomic DNA (extracted from individual F2 embryos) reveal the absence of homozygous mutants.

In order to assess whether *gas6*, *gbx1*, *sall4*, *egfl6*, *celf2* and *greb1l* are required for rhombomere formation, we assayed expression of *hoxb1a* in r4, *krox20* in r3 and r5, *pax2* at the MHB boundary and in the otic vesicle, as well as of *hoxd4a* in r7 and anterior spinal cord, by ISH in each of the mutant lines. Upon breeding the mutant lines, we noted that the *gas6*, *sall4*, *greb1l* and *egfl6* lines produced homozygous mutants at close to the expected ratio, while the *celf2* and *gbx1* lines produced few, or no, homozygous mutant offspring (Table 3.2). This is in agreement with previously published information where *gbx1* mutants obtained via ENU mutagenesis, were also not viable as homozygotes¹⁶⁵. We also find that homozygous *gas6*, *sall4*, and *greb1l* mutants are fertile, but homozygous *egfl6* mutants are not. Therefore, our functional analyses made use of offspring from crosses of homozygous mutant parents for *gas6*, *sall4* and *greb1l*, but offspring of heterozygous carriers for the other lines. Strikingly, we do not detect gene expression changes in any of the mutants (Figure 3.14).

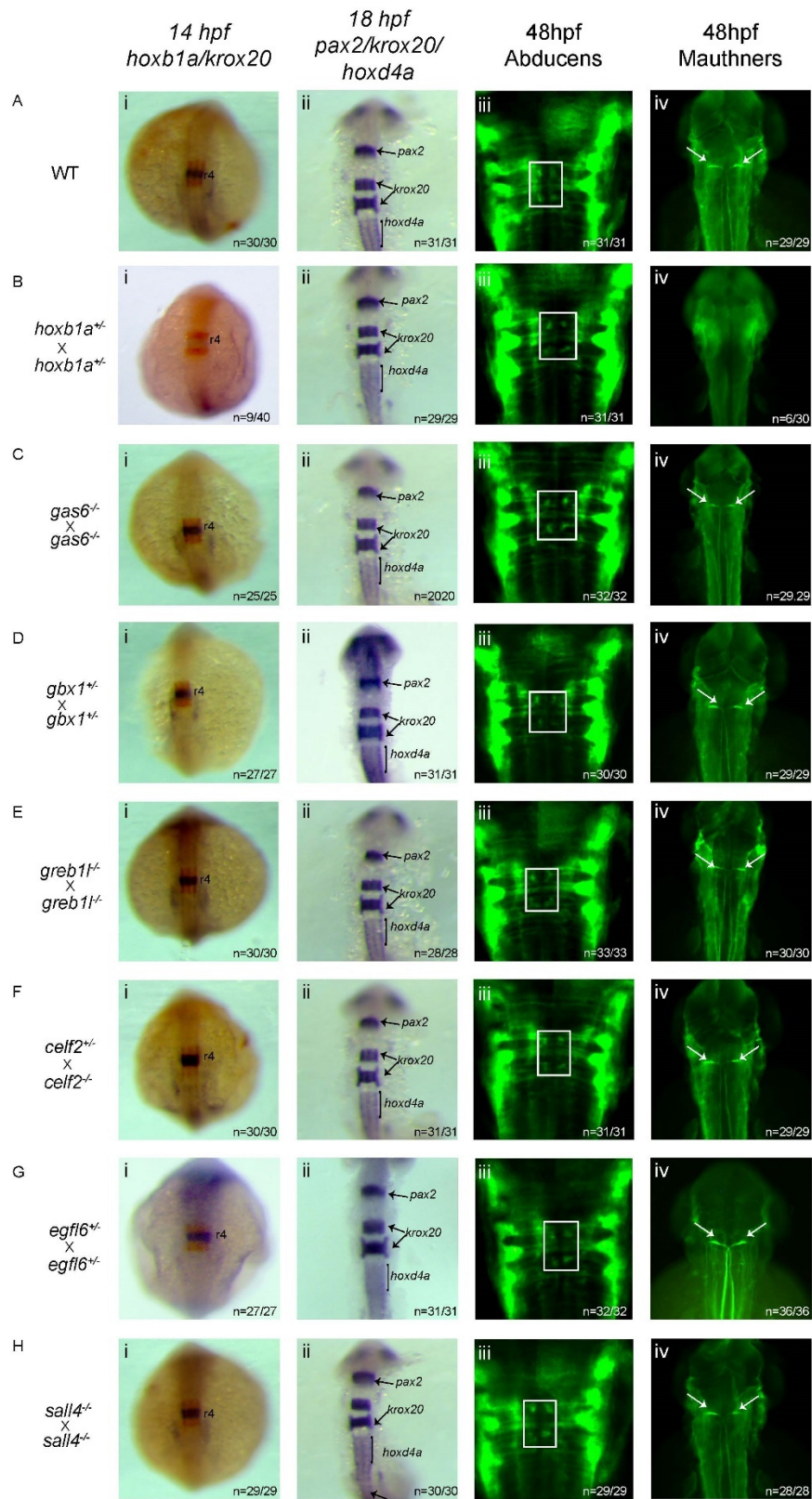


Figure 3.14 *gas6*, *gbx1*, *sall4*, *egfl6*, *cellf2* and *greb1l* function is not required for r4-r6 formation.

ISH for hindbrain markers (i) *hoxb1a* (blue, r4) and *krox20* (red r3/5), (ii) *pax2* (MHB), *krox20* (r3/5) and *hoxd4a* (r7-anterior spinal cord), and immunostaining for neuronal markers detecting (iii) abducens motor neurons (four green dots in white boxes) in r5/6 and (iv) Mauthner neurons (white arrows) in r4 was carried out on embryos collected from an (A) cross of WT fish, (B) cross of *hoxb1a* heterozygous mutants, (C) cross of *gas6* homozygous mutants, (D) cross of *gbx1* heterozygous mutants, (E) cross of *greb1l* homozygous mutants, (F) cross of a *cellf2* heterozygous and a homozygous mutant, (G) cross of *egfl6* heterozygous mutants and (H) cross of *sall4* homozygous mutants. All embryos are oriented in dorsal view with anterior to the top. Embryos collected at 14hpf and 18hpf were imaged as whole-mounts. 48hpf embryos were flat-mounted for imaging.

We also do not detect any defects in rhombomere size, in the spacing of the rhombomere expression domains, or in the integrity of rhombomere boundaries. As a further test of rhombomere development, we also examined the differentiation of rhombomere-specific neurons. Specifically, we used immunochemistry to visualize reticulospinal Mauthner neurons in r4 (Figure 3.14, column iv) and nVI abducens neurons in r5/6 (Figure 3.14, column iii). Results from this analysis revealed the presence of normal and properly patterned neurons in each of the mutants. Hence, our results indicate that the *gas6*, *gbx1*, *greb1l*, *celf2*, *egfl6*, and *sall4* genes are not required for rhombomere-restricted gene expression or neuronal differentiation in the zebrafish hindbrain.

A detailed analysis of *gas6* mutants does not reveal hindbrain defects

We next considered the possibility that the mutant lines may have subtle phenotypes that went undetected by our initial screening. To test this possibility, we took advantage of the viability of the *gas6* homozygous mutants and selected it for in-depth analysis. Since *krox20* expression is unaffected in *gas6* (Figure 3.14Ci and ii) mutants, we reasoned that *gas6* might act downstream of *krox20* and therefore examined expression of two later-acting r5/r6 genes (*hoxb3a* and *hoxa3*). However, we find that expression of both genes is unaffected in *gas6* mutants (Figure 3.15Ai and ii). We also examined the migration of nVII facial motor neurons from r4 into r5/r6 (Figure 3.15Aiv, blue bracket) but do not detect

any disruptions of this process in *gas6* mutants. Lastly, r5 and r6 are the source of the initial wave of oligodendrocyte precursor cells

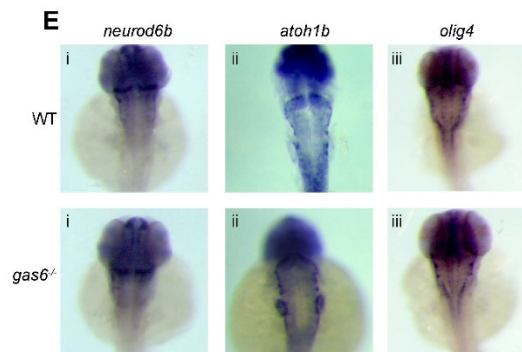
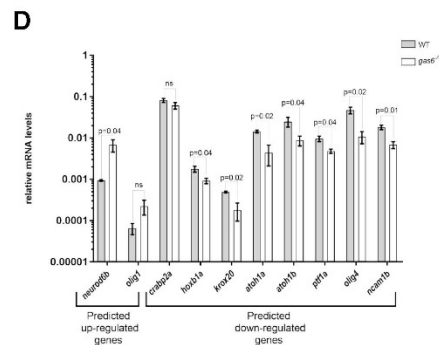
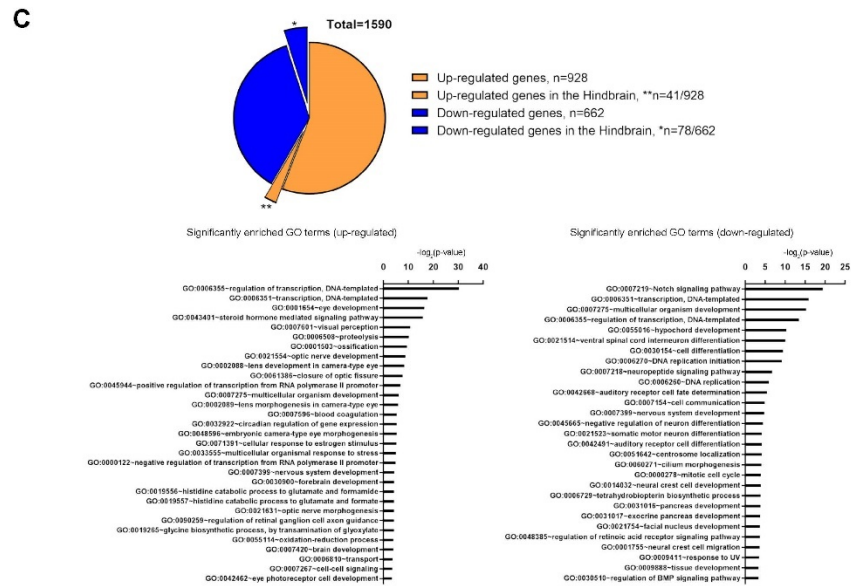
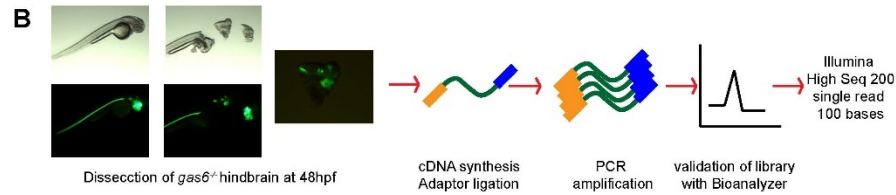
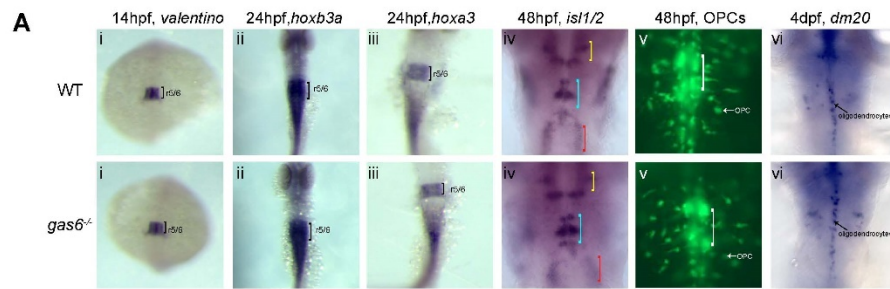


Figure 3.15 *gas6* may only have subtle roles in caudal hindbrain development.

(A) WT and *gas6* mutant embryos were assayed for expression of *valentino* (r5/6) (i), *hoxb3a* (r5-spinal cord) (ii), *hoxa3* (r5/6) (iii), *islet1* (cranial nerves) (iv) and *dm20* (oligodendrocyte marker) (vi) by ISH, as well as for the presence of OPCs and Abducens neurons by crossing to the *Tg(olig2:EGFP)^{vu12}* line (v). In column (iv), yellow brackets mark cranial nerve V, blue brackets mark cranial nerve VII and red brackets mark cranial nerve X. White brackets indicate the presence of Abducens (cranial nerve VI) in column (v). (B) Schemes showing RNA-seq library synthesis. Hindbrain tissue was dissected from 48 hpf *gas6* mutant embryos in the *olig2:eGFP* background. Total RNA was collected from pools of hindbrain tissue and was used in library synthesis following the TruSeq Stranded mRNA Library Prep Kit (Illumina) protocol. (C) 1590 differentially expressed genes were identified from RNA-Seq where 41 out of the 928 up-regulated genes and 78 out of the 662 down-regulated genes were expressed in the hindbrain. GO terms related to Biological Processes were identified in both up-regulated and down-regulated genes using DAVID. (D) A subset of differentially expressed genes was validated via qPCR from independently collected hindbrain tissue samples. (E) ISH analysis of representative differentially expressed hindbrain genes (i) *neurod6b*, (ii) *atoh1b* and (iii) *olig4* show no detectable change in expression pattern in *gas6* mutant embryos.

(OPCs) (Figure 3.15Av) in the hindbrain and we therefore examined expression of *olig2* (a gene required for OPC formation) and *dm20* (a marker of differentiated, myelin-producing oligodendrocytes) (Figure 3.15Avi) in *gas6* mutants, but do not find oligodendrocyte formation to be affected in *gas6* mutants.

For a more global view of potential defects in *gas6* mutants, we used RNA-seq to compare gene expression between homozygous *gas6* mutants and wildtype embryos. Since *gas6* is expressed exclusively in the hindbrain, we made use of dissected hindbrains from wildtype and *gas6* mutants at 48 hpf (Figure 3.15B). Our analysis identified 1,590 genes with a 2-fold or greater change in expression between wildtype and mutant hindbrains (928 up-regulated and 662 down-regulated in *gas6* mutants) (Figure 3.15C). Subsequent RT-qPCR analysis on ten differentially expressed genes (*olig1*, *neurod6b*, *crabp2a*, *hoxb1a*, *krox20*, *atoh1a*, *atoh1b*, *ptf1a*, *olig4* and *ncam1b*) confirmed the gene expression changes observed by RNA-seq (Figure 3.15D). Using the DAVID functional annotation tool ¹⁶⁶, we find enrichment for genes associated with developmental processes like “nervous system development”, “forebrain development”, and “neural crest development”, but only a few genes are associated with each GO term. Furthermore, comparison to the hindbrain-expressed genes identified in our database search revealed that only ~7.5% of the genes differentially expressed in *gas6* mutants (41 up-regulated and 78 down-regulated; Figure 3.15C) are expressed in the hindbrain. However, when we use ISH to assess

expression of three of these genes (*neurod6b*, *atoh1b* and *olig4*), we do not detect any differences between wildtype and *gas6* mutant embryos (Figure 3.15E). We conclude that disruption of *gas6* leads to changes in hindbrain gene expression, but these changes are too subtle to be detected by ISH and do not affect rhombomere formation or neuronal patterning.

CHAPTER IV: DISCUSSION

Our goal for this study was to identify novel genes required for caudal hindbrain development and to position them within the corresponding GRN (Figure 4.1). We used the ZFIN database to identify 84 genes that are expressed in r4-r6, but that are relatively uncharacterized. We focused on 22 representative genes and find important differences between r4 and r5/r6 gene expression. In particular, we find that r4 genes are under the combinatorial regulation of RA and *hoxb1b* while r5/r6 genes are under control of RA, FGF, *hnf1ba* and *valentino* in a regulatory arrangement where the loss of any one of these factors disrupts r5/r6 gene expression. Additionally, we identified several novel interactions between the r4 and r5/r6 gene-sets. This includes the repression of *dusp6*, *spry1* and *egfl6* by *hnf1ba* and repression of *gbx1* by *hoxb1a* (Figure 4.1). We also analyzed germline mutants for six genes (*gas6*, *gbx1*, *sall4*, *eglf6*, *cellf2*, and *greb1l*), but we do not detect hindbrain defects in any of the mutants. However, transcriptome profiling of *gas6* mutants identified differentially expressed genes involved in a variety of hindbrain related developmental processes—leading us to speculate that *gas6* may play subtle roles in hindbrain development. Thus, our study suggests that the regulatory logic differs in r4 versus r5/r6, but that both GRNs are relatively robust with only few genes being absolutely required for their integrity. In the following sections we will discuss the implications of our findings, address the limitations in this study, and propose future experimental ideas.

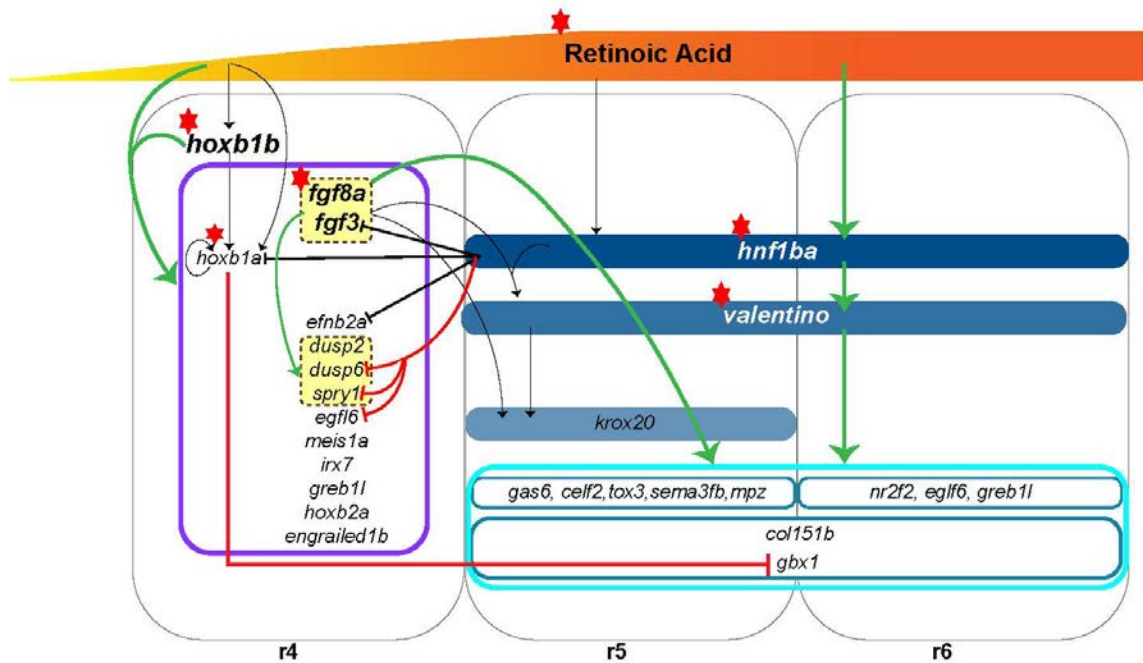


Figure 4.1 Proposed model depicting the GRN in the caudal hindbrain.

Black arrows and bars represent regulatory relationships known prior to this study. Green arrows and red T bars represent relationships uncovered in this study. In this model, arrows (activating) and T bars (repressive) indicate interactions that have been observed, they do not indicate whether the interactions are direct or indirect. All r4 genes regulated by *hoxb1b*+RA are grouped in the purple box. Within r4, *dusp2*, *dusp6* and *spry1* are regulated by FGF signaling (yellow box). *hnf1ba* represses *dusp6*, *spry1* and *egf6* expression while *gbx1* expression is repressed in r4 by *hoxb1a*. All r5/r6 genes (light blue box) are regulated by RA, FGF, *hnf1ba* and *valentino*. Red star next to RA, FGFs, *hoxb1b*, *hoxb1a*, *hnf1ba* and *valentino* represent the key regulators of the caudal hindbrain – without these factors r4-r6 does not form properly.

The r4 and r5/r6 gene regulatory networks operate by different mechanisms

Previous studies demonstrated that loss of PG1 *hox* function results in a mis-specified r4^{17,45,52,62,64,110,162,167}, leading us to hypothesize that all r4 genes are regulated by PG1 *hox* genes. Surprisingly, our ISH analysis of the r4 gene-set showed no significant change in expression in PG1 *hox* mutants. While a recent report indicates that expression of FGF pathway components is reduced in *hoxb1b* mutants⁶², we do not observe such an effect, possibly due to differences in sensitivity of the ISH protocols used. Additionally, while our transcriptome analysis of *hoxb1b* mutants identified differentially expressed genes present in the hindbrain, the expression changes were relatively subtle, owing mostly to the reduction in the size of r4. These results suggest that r4 gene regulation may require other factors in addition to PG1 *hox* genes. Indeed, we observe complete loss of expression of all tested r4 genes when *hoxb1b* and RA function is simultaneously disrupted, demonstrating that both factors are required to control r4 gene expression.

In r5/r6, the available data predict a relatively linear pathway where RA, FGF, *hnf1ba* and *valentino* control r5/r6 identity and disruption of any one of these factors causes r5/r6 defects. Additionally, at least in mice, r5 cells adopt an r6 fate in the absence of *krox20*^{98,107} and combined mutations in the mouse PG3 *hox* genes *Hoxa3* and *Hoxb3* result in loss of r5/r6 specific abducens motor neurons⁶⁹, suggesting that *krox20* and PG3 *hox* genes are also required for r5/r6 formation. In accordance with the prevailing model, we find that expression of all

tested genes from the r5/r6 gene-set is abolished in *hnf1ba* and *valentino* mutants, as well as upon disruption of RA or FGF signaling. Notably, there are combinatorial interactions also in r5/6 – for instance, *hnf1ba* and FGF act together to drive *valentino* expression^{85,87} – but the mechanism of combinatorial regulation differs between r4 and r5/r6. In r4, *hoxb1b* and RA function together to drive gene expression and either factor is sufficient to support expression. However, in r5/r6, neither *hnf1ba* nor FGF is sufficient to support r5/r6 gene expression. Hence, the r4 GRN appears less susceptible to disruptions than the r5/r6 GRN (Figure 4.1). It is not clear why this would be the case, except that r4 is the earliest rhombomere to form and it acts as a key signaling center during hindbrain development, raising the possibility that there may have been greater evolutionary pressure to ensure that r4 forms properly.

Speculations about the regulation of r4 genes by *hoxb1b* and RA

The most interesting observation in this study is the loss of all candidate r4 gene expression due to the elimination of RA in *hoxb1b* mutants. However, the details underlying this combinatorial regulation remain unclear. Recent studies have shown that RA signaling is unaffected by loss of PG1 *hox* function⁶²; thus, it is likely that these factors act in parallel and may even compensate for one another.

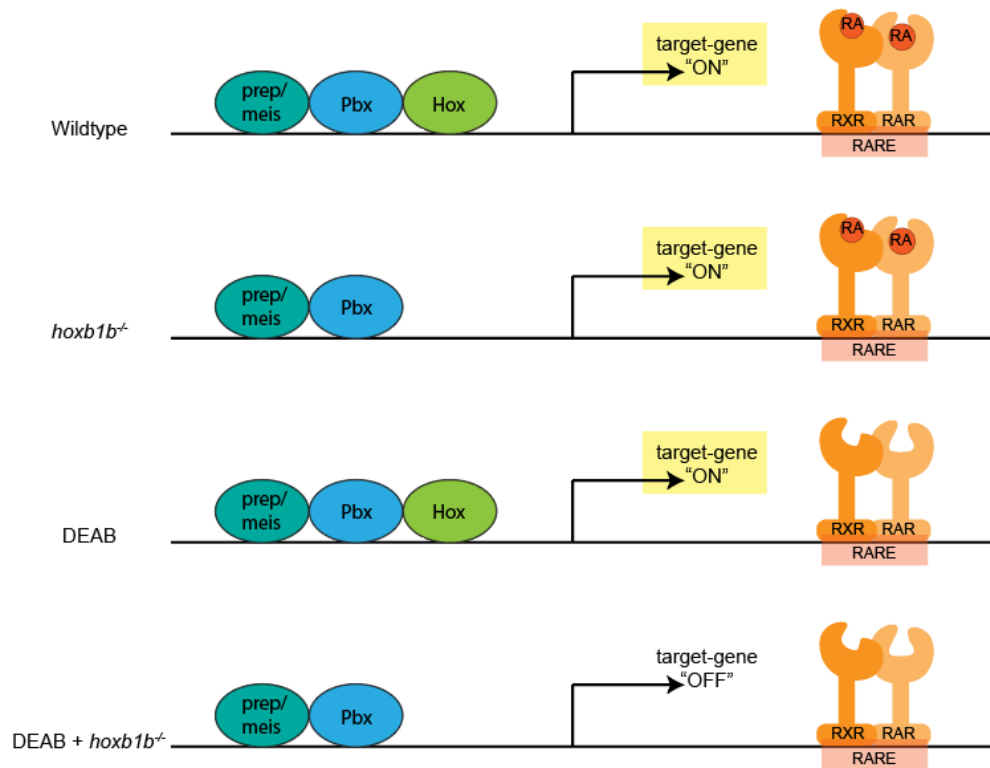


Figure 4.2 Speculations on r4 gene regulation by *hoxb1b* and RA.

Using *hoxb1a* as a model for all target r4 genes we can speculate about these genes are controlled by the combinatorial regulation of *hoxb1b* and RA. In this figure, the RARE element is shown 3' to the target genes, and Hox: Prep/Meis: Pbx site is 5' to the gene—this is the case for *hoxb1a* and thus is being used as an example. It is worth noting that RARE and Hox binding sites can be present on either end of the target gene, and also in either the promoter or enhancer region. Under wildtype conditions, both RA and *hoxb1b* drive gene expression. The presence of any one of these regulators is sufficient to drive gene expression. Loss of both *hoxb1b* and RA will turn “off” gene expression.

However, it is unknown if our candidate genes have Hox binding sites and RAREs. At least for one gene, *hoxb1a*, we know that there is a 3' RARE element and bona fide Hox (Hoxb1b), Prep/Meis and Pbx binding site in its promoter; demonstrating that *hoxb1a* indeed has sites for *hoxb1b* and RA mediated regulation^{73,109}. Simplistically, it seems that *hoxb1a* has two regulatory options: a Hox-dependent or a RA-dependent mechanism. Either is sufficient to drive *hoxb1a* expression; simultaneous elimination of *hoxb1b* and RA leads to loss of *hoxb1a*. Considering that all candidate r4 genes behave like *hoxb1a* (i.e. - expression is lost only when both RA and *hoxb1b* are absent), we could hypothesize that r4 genes have two 'triggers' that can co-operate to instigate gene expression (Figure 4.2). Needless to say, we are assuming that the candidate r4 genes will all have Hox binding sites and RAREs. It would be interesting to follow-up on this by performing ChIP analyses to locate such sites. Currently, an effort to identify Hox: Prep/Meis: Pbx sites is in progress. The possible presence of both regulatory sites raises another question—in wildtype conditions, is it necessary to have both these sites (Hox binding/RARE) occupied to drive gene expression? Site-directed mutagenesis of the Hox binding sites and RAREs could potentially help us answer such a question—mutations in both regulatory locations should phenocopy DEAB treated *hoxb1b*^{-/-} hindbrains. These speculations are being made based on the idea that RA and *hoxb1b* acts directly at each r4 gene; it is probable that an intermediary factor is required to drive r4 gene expression.

Elimination of RA signaling in *hoxb1b* mutants affects *krox20* expression in r3

In the zebrafish hindbrain, *krox20* is expressed in r3 and r5 but it is regulated through separate mechanisms in these two rhombomeres. In r5, FGF (via element C), *hnf1ba*, and *valentino* (via element B) initiate and maintain *krox20* expression. As shown in this study (and previously published work), blocking any one of these factors leads to the loss of r5 restricted *krox20*^{14,17,19,40}. This is in line with the fact that FGFs are caudalizing morphogens and that *hnf1ba* and *valentino* specify the identity of r5—thus, improper r5 specification leads to loss of *krox20* expression in r5.

The regulation of *krox20* in r3 is less understood. Blocking RA signaling never affects r3 *krox20* expression¹⁷. In early stages of development, *hoxb1a* initiates *krox20* but later on *hoxb1a* will repress it^{101,168,169}. However, our data shows that the loss of *hoxb1a* has no effect on *krox20* expression. One possibility is that TFs in the Irx and Meis family (Figure 4.3) may play a role in controlling *krox20* expression in r3. Morphants for *irx7* and *irx1b* lack *krox20* expression specifically in r3. Combined over-expression of *irx7* and *meis* leads to ectopic *krox20* expression in the anterior neural plate—suggesting that *irx7* may regulate *krox20* in r3 in a *meis* dependent manner⁹⁵.

In this study we see that blocking RA in *hoxb1b* mutants not only leads to the loss of the all r4 candidate genes it also abolishes *krox20* expression in r3. This is a striking observation because, for the first time, we demonstrate that the

combined loss of RA and *hoxb1b* affects the expression of genes anterior to r4. As a consequence of blocking RA in *hoxb1b* mutants we effectively lose the expression of *hoxb1a*, *irx7* and *meis1a*, all of which explains the loss of *krox20* in r3. However, the exact mechanisms by which these genes regulate *krox20* expression in r3 remains unknown. The absence of *krox20* in r3 due to the simultaneous elimination of *hoxb1b* and RA could be indicative of the fact that the entire hindbrain is mis-specified leading to an expansive loss of gene expression.

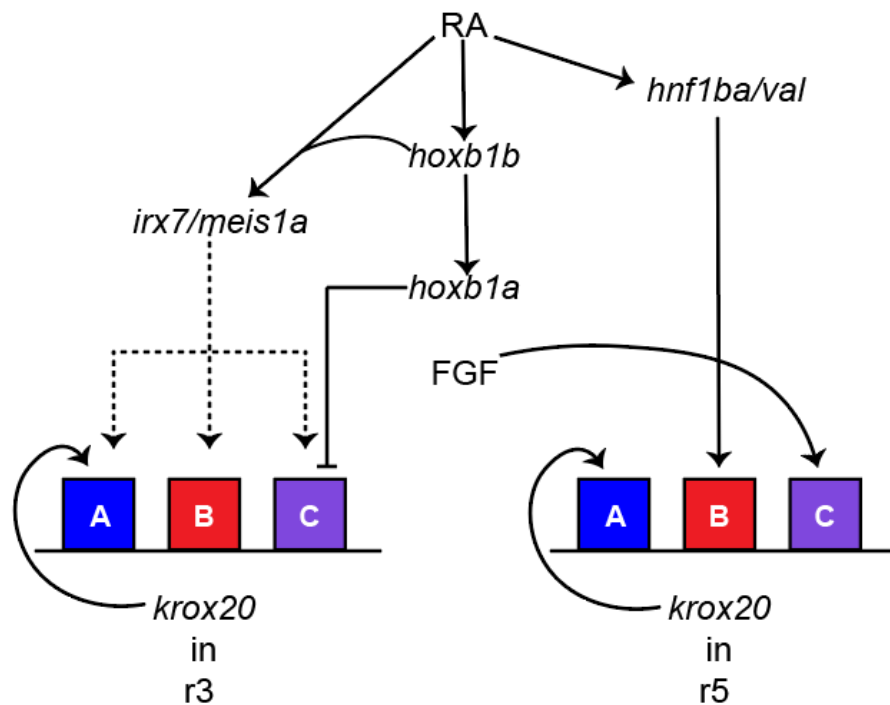


Figure 4.3 Differential regulation of *krox20* in r3 and r5.

Elements A (blue box), B (red box), C (purple box), play a role in initiating and maintaining *krox20* expression. In r5, FGF, *hnf1ba* and *valentino* control *krox20* expression. In r3, we hypothesize that *irx7* and *meis* genes (dotted lines) regulate *krox20* expression in a RA/*hoxb1b* dependent manner.

Combined loss of RA and *hoxb1b* reverts the hindbrain to its “groundstate”

Prior to this study (and subsequently confirmed by our experiments) RA has been shown to maintain and establish the caudal identity of the hindbrain. Loss of RA signaling (both DEAB treatment and *raldh2* hypomorph mutants) shows an enlargement of r3 with no effect on r3 gene expression, a slight enlargement of r4, and a complete loss of r5/6 genes^{14,17,20}. Mutations in *hoxb1b* alone affects the size but not the expression of genes in r4^{62,64}. In this study we demonstrate that the combined loss of RA and *hoxb1b* leads to a severe phenotype affecting the integrity of the hindbrain. The lack of gene expression in the entire hindbrain closely resembles the phenotype seen due to the loss of *pbx2/4*¹⁷⁰ (Table 4.1). Closer observation of our data show that a few genes which are expressed in r1/2; such as *fgf8a* and *eglf6* (Figure 3.6 Fiv and Kiv), show a caudal expansion into the hindbrain when RA is blocked in the *hoxb1b* mutants. This is similar to the posterior expansion of r1 genes *fgfr3* and *epha4a* in *MZl/r*; *pbx2*MO fish (*pbx2* morpholino injected into a maternal zygotic *pbx4*^{-/-} mutant). It is also worth noting that loss of *pbx2/4* resulted in the loss of *krox20* (in both r3 and 5), *hoxb1a*, and *valentino* expression —our results mirror these observations. This condition, where there is a global loss of rhombomere identity has been described as a “groundstate”¹⁷⁰ (Table 4.1). Prior studies have shown that RA can up-regulate the expression of proteins in Pbx family, and in turn, both Hox and Pbx proteins regulate RA expression (via regulating *raldh2* transcription)^{62,171,172} (Table 4.2). Additionally, recent studies show that loss of

both *pbx4* and *hoxb1b* regresses the hindbrain to this r1-like “groundstate”⁶² (Table 4.1). Thus, our observation that DEAB+*hoxb1b*^{-/-} fish have hindbrains that have reverted back to this “groundstate”, is in line with the existing information about the relationship between Hox, Pbx and RA (Figure 4.4). In keeping with the theme of our observations, we see that not all r1/2 genes behave the same way. While the anterior domains of *fgf8a* (r1/2) and *egfl6* (r2) show a posterior expansion, the r2 domain of *greb1l* does not (Figure 3.6 Jiv). Once again, this demonstrates that the regulatory logic will often vary from gene to gene. To truly determine whether the DEAB treated *hoxb1b* hindbrain reverts to its groundstate, we would need to perform transcriptome analysis. Comparisons to wildtype hindbrains or even to dissected portions of it (i.e. dissected r1/2) would help us understand if indeed the hindbrain has transitioned to its groundstate. It is worth mentioning that at the time of writing this discussion, such a transcriptome analysis (comparison between wildtype and DEAB+*hoxb1b*^{-/-}) is in progress in our lab.

Table 4.1 Genetic losses that revert hindbrain to “groundstate”

Model Organism	Genetic manipulation	Affect
Zebrafish	MZlzl ^{-/-} + <i>pbx2</i> MO	No rhombomere boundaries, No <i>krox20</i> , <i>hoxb1a</i> and <i>valentino</i> expression, posterior expansion of <i>fgfr</i> and <i>epha4a</i> ¹⁷⁰
	<i>pbx4</i> ^{-/-} ; <i>hoxb1b</i> ^{-/-} double mutant	No <i>krox20</i> expression, posterior expansion of <i>epha4a</i> ⁶²
	DEAB treated <i>hoxb1b</i> ^{-/-} embryos	Loss of all hindbrain genes with posterior expansion of r1/2 domains of <i>fgf8a</i> and <i>egf16</i>
	<i>hoxb1b</i> ^{-/-} ; <i>hoxb1a</i> ^{-/-} double mutant	No major loss of gene expression*; <u>hindbrain does not revert groundstate</u> ⁶²

* subtle changes in *hoxb2a*, *fgf8a*, *fgf3*, *dusp6*, and *spry1* expression.

Table 4.2 Review of the role of Pbx and Hox in regulating RA

Model Organism	Genetic manipulation	Effect on <i>raldh2</i>
Mouse	<i>Hoxa1/Pbx1</i> null mice ¹⁷²	reduction in <i>raldh2</i>
	<i>Pbx1/Pbx2</i> null mice ¹⁷²	
Zebrafish	<i>pbx2/4</i> morphant embryos ¹⁷³	
	<i>pbx4</i> ^{-/-} + <i>pbx2/4</i> MO ⁶²	No effect on <i>raldh2</i>
	<i>hoxb1b</i> ^{-/-} ; <i>hoxb1a</i> ^{-/-} double mutant ⁶²	

Alternative mechanisms regulating RA and *hoxb1b* expression

The current study has re-enforced the importance of both RA and *hoxb1b* in regulating genes in the hindbrain; however, some aspects of how RA and *hoxb1b* expression is initiated are unclear. It has been shown that RA regulates *hoxb1b*^{31,174}; if so, we should have seen a complete loss of *hoxb1b* when RA signaling is eliminated. However, several studies have shown that this is not the case. Both DEAB treated wildtype embryos and *raldh2* hypomorph mutants retain low levels of *hoxb1b* expression^{17,19,36}. One possibility is that there are remnants of low level RA signaling that is sufficient to initiate *hoxb1b* transcription. More recently, an alternative path, where a zinc transcription factor called *znfl1* has been shown to regulate *hoxb1b*. Moreover, *znfl1* is expressed from early stages of development in the posterior neuroectoderm, an expression pattern comparable to *raldh2* expression¹⁷⁵. Further studies should be performed to determine the relationship between *znfl1* and RA. It is also unknown how RA signaling is initiated. While Pbx and Hox proteins are proposed regulators of RA^{172,173} (Table 4.2), additional genes like *zic2a*, *zic2b*, *tgif*, and *hmx4* are also suggested to regulate the expression of RA¹⁷⁶. Such observations validate the complexity of GRNs and shows that additional analysis is necessary to fully understand the regulatory connections between genes (Figure 4.4).

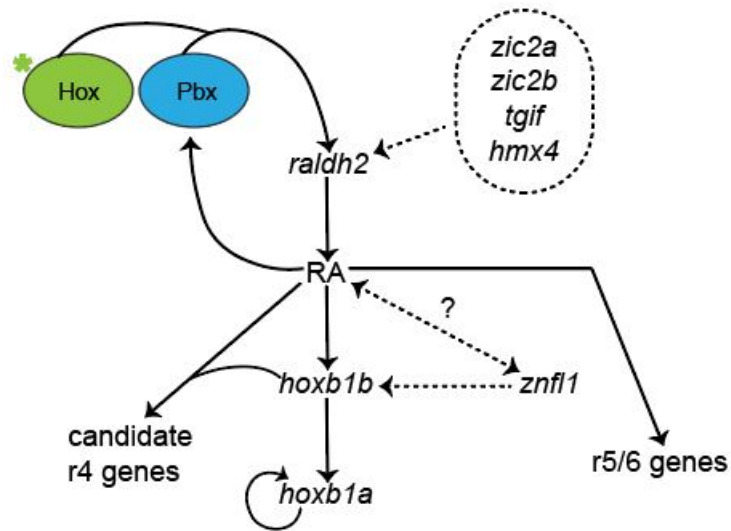


Figure 4.4 Alternative mechanisms of RA and *hoxb1b* regulation

RA can up-regulate Pbx proteins which in turn can control RA signaling via regulating the transcription of *raldh2*. Hox proteins (*only in mice) are also shown to regulate RA signaling. *znfl1* is considered as an alternative source of *hoxb1b* regulation. *zic* transcription factors, *tgif*, and *hmx4* have also been suggested regulators of RA signaling. Combinatorial losses in Pbx, RA and *hoxb1b* can revert the hindbrain to a “groundstate”.

Repressive interactions may represent a key function of the hindbrain

GRNs

Cross-talk between r4 and r5/r6 genes is a crucial part of establishing rhombomere boundaries and maintaining the uniqueness of each rhombomere (Figure 4.5). An example of this is seen in *hnf1ba* mutants where there is posterior expansion of the r4 genes *hoxb1a*, *fgf3*, and *efnb2a* into the mispatterned r5/r6 domain⁸⁵. In this study, we identified *dusp6* (Figure 3.2Civ), *spry1* (Figure 3.2Div), and *egl6* (Figure 3.2Miv) as additional r4 genes whose expression domains are defined by *hnf1ba*-mediated repression. Importantly, *hnf1ba* is thought to act primarily as a transcriptional activator¹⁷⁷, raising the possibility that *hnf1ba* controls expression of a transcriptional repressor in r5/r6. Such an indirect effect may be mediated by *krox20*, which represses *Hoxb1* (murine ortholog of zebrafish *hoxb1a*) expression in r4^{103,104,178–180}. In particular, *krox20* activates the expression of Nab proteins, which are known negative regulators of transcription – making them possible candidates for mediating the effect of *hnf1ba* in repressing r4 gene expression¹⁸¹. Furthermore, the fact that only a subset of r4 genes is repressed by *hnf1ba*, suggests that additional factor(s) might be responsible for repressing the remaining r4 genes in an *hnf1ba*-independent manner (Figure 4.5).

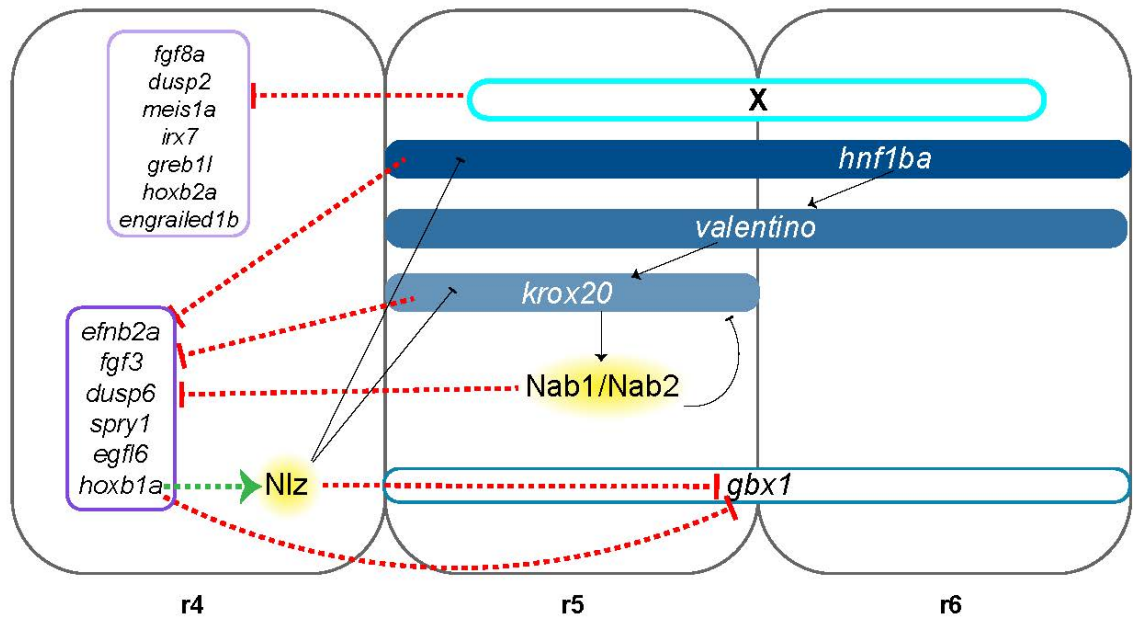


Figure 4.5 Repressive interactions between r4 and r5/6 genes in the zebrafish hindbrain. Black arrows and bars represent interactions known prior to our study. Dotted arrows and bars are hypothesized interactions between r4 and r5/6 genes. *hnf1ba* represses a subset of r4 genes (dark purple) either directly (unlikely since it is not known as a transcriptional repressor) or indirectly by activating *krox20*. *krox20* could repress the subset of r4 genes directly or could do so via Nab proteins. The remaining r4 genes (light purple) that are probably regulated by factor “X” in an *hnf1ba* independent manner. *hoXB1a* either directly or indirectly (via Nlz) represses *gbx1* expression in r4.

We did not detect a reciprocal role for PG1 *hox* genes in repression of r5/r6 gene expression, but our experiments did demonstrate *hoxb1a*-mediated repression of *gbx1* in r4 (Figure 3.8Hiii and Figure 3.10Ai). We do not know the mechanism for this repression, but it may be indirectly mediated by Nlz proteins – members of a subfamily of zinc-finger proteins. Previous work demonstrated that Nlz proteins, which are found in r4, act as transcriptional repressors and *n lz* loss of function leads to gene expression from adjacent rhombomeres expanding into r4^{168,182–186}. Since *n lz1* expression is regulated by PG1 *hox* genes¹⁶⁸, *gbx1* repression may be indirectly mediated by *hoxb1a* via Nlz proteins.

Members of the r4 and r5/r6 gene sets are not essential for hindbrain development

To test if members of the r4 and r5/r6 gene sets regulate caudal hindbrain formation, we analyzed germline mutants for six genes (*gas6*, *gbx1*, *sall4*, *eglf6*, *cellf2*, and *greb1l*). Our results reveal that in all of these mutant lines, hindbrain patterning and the subsequent development of the r4 specific Mauthner neurons, and the r5/r6 specific Abducens neurons are all normal. Detailed transcriptome analysis of *gas6* mutants identified differentially expressed genes involved in neuronal development (discussed in the detail in the following section), but the expression changes are subtle and cannot be detected by ISH. Hence, these genes do not appear to be essential for hindbrain development. We cannot fully exclude the possibility that some residual gene activity persists in the specific

mutants assayed. For instance, the *egfl6* mutation affects a splice junction and some mutants may also harbor maternal transcripts or proteins. However, in three cases (*gas6*, *sall4* and *greb1l*) where we were able to assay the offspring of homozygous mutant parents, which eliminates the concern with maternal products. Furthermore, the viability of homozygous *gbx1* and *cellf2* mutants was reduced, while *egfl6* homozygous mutants were infertile, demonstrating that these genes are important, just not for hindbrain development. Based on these analyses, it appears that most members of the r4 and r5/r6 gene sets may not be individually essential for hindbrain development. Accordingly, we recently found that *dusp6* and *dusp2* homozygous mutants also have normal hindbrain and neuronal patterning³⁵. Hence, our data suggest that caudal hindbrain development is robust, and genes involved in this process most likely have redundant roles such that the loss of a single gene will not cause gross developmental defects.

Putative role of *gas6* in regulating gliogenesis and myelination

In this study, we have characterized *gas6* as a gene that is expressed in rhombomeres 5 and 6 and is under the regulation of the RA, FGF, *hnf1ba*, and *valentino* signaling network. The only known role of *gas6* in zebrafish is angiogenesis in retinal tissue¹⁸⁷. Therefore, it was of interest to determine the role of this rhombomere-restricted gene in the zebrafish; specifically, in the hindbrain.

gas6 is a vitamin-k dependent soluble growth factor and is widely expressed throughout the nervous system. It was cloned following its up-regulation in confluent cell lines where it protected cells from death ^{188,189}. *Gas6* is a ligand for the receptor *Axl*, a member of the TAM subfamily of receptor tyrosine kinases. *Gas6/Axl* interaction can activate a myriad of signaling pathways including the ERK, PI3K/Akt, and JAK/STAT pathway. These signaling events lead to cell proliferation, cell survival, immune cell differentiation cell adhesion, and cell migration ^{189–192}.

Studies performed in mice and cultured human cells have shown that *Gas6* is involved in gliogenesis and myelination. Following cuprizone mediated demyelination, introduction of soluble *Gas6* increases myelination in mice with a dose dependent increase of MBP+ (myelin basic protein) oligodendrocytes. Furthermore, *Gas6*^{-/-} mice show reduced remyelination following cuprizone treatments ^{191,193,194}. In human fetal dorsal root ganglions co-cultured with OPCs (oligodendrocyte progenitor cells), exposure to soluble *Gas6* once again showed an increase in of MBP+ oligodendrocytes and resulted in thicker axonal myelin sheaths ¹⁹⁵. Another study in mice showed that *Gas6* activated the JAK/STAT to promote oligodendrogenesis and myelination following lysolecithin induced demyelination ¹⁹⁶. More recent work shows that *Gas6/Axl* double knockout mice have motor deficits and reduced remyelination ¹⁹⁷ following cuprizone mediated demyelination.

The primary goal of this study was to determine if *gas6* affects hindbrain patterning in zebrafish. Since hindbrain OPCs originate from the r5/6 region, the same region where *gas6* is expressed, it would be logical to look at oligodendrocyte development. Crossing the transgenic line *Tg(olig2:EGFP)^{vu12}*¹⁵⁵ into the *gas6* mutant background enabled us to visualize Olig2+ OPCs in mutant background. However, considering the above discussion, it was interesting to observe that our *gas6* mutant zebrafish had apparently normal OPCs and mature oligodendrocytes. At 48 hpf, the mutants are comparable to the wildtype fish in terms of proliferating OPCs. Similarly, at 4 dpf, both mutant and wildtype embryos seem to have the same number of mature oligodendrocytes. However, our studies had several drawbacks, one being the time at which the OPCs and oligodendrocytes were studied. Most OPCs are specified and detected in the hindbrain around 24 hpf. By 48 hpf OPCs begin to proliferate and migrate. Mature oligodendrocytes will produce nascent myelin around 3 dpf and the first compact myelin sheaths are not visible till after 7 dpf^{155,198}. Thus, it is possible that at later developmental timepoints, we might have noticed some defects. Another shortcoming in our study was the type of microscopy used. Our observations were made using brightfield microscopy which has a small range of magnification and captures a single focal plane. These limitations could have lead us to falsely conclude that *gas6* mutants do not have defective OPCs/oligodendrocytes. Furthermore, we never studied myelination. To do so, the *Tg(mbp:egfp)* line can be crossed into the *gas6*

mutant background-enabling us to visualize myelin (GFP+ cells). In accordance to methods published by Jung and colleagues ¹⁹⁹, confocal imaging of transverse hindbrain and spinal cord sections would allow us to measure the g-Ratio (index of optimal axonal myelination) ²⁰⁰ and determine if the loss of *gas6* affects myelination. For more in-depth analysis, a combination of tissue clearing, antibody labeling, transgenic lines, and light sheet confocal imaging could be used to create a 3-dimensional documentation of OPC proliferation, oligodendrocyte development and myelination ^{201,202} in *gas6* mutant zebrafish.

While pursuing gliogenesis and myelination were beyond the scope of this project, transcriptome analysis of *gas6* mutants reveal information that connects *gas6* to its potential role in myelination. From the RNA-seq data, we see that the most significantly enriched GO term (in down-regulated differentially expressed genes) is associated with Notch signaling. DAVID analysis shows that Notch signaling pathway associated genes - *dla*, *dlb*, *dld*, *jag2b*, *notch1a*, *nrarpb*, *lfng*, *her2*, and *her12* are all significantly reduced in *gas6* mutants (Table 4.3).

Notch signaling plays a fundamental role in both gliogenesis and myelination in the nervous system. Notch signaling is triggered by direct contact between the receptor and its ligand. Classic Notch ligands include members of the Jagged and Delta family. Ligand binding leads to the cleavage of the intracellular form of Notch followed by subsequent translocation into the nucleus. In the nucleus, this intracellular form of Notch transcriptionally regulates genes

Table 4.3 Differentially expressed genes involved in Notch signaling in *gas6*^{-/-} hindbrain

Down-regulated genes			
associated with the Notch pathway	log2FoldChange	Fold change	p-adj
<i>nrarpb</i>	-1.096359485	0.467695196	1.73E-21
<i>dla</i>	-1.10766798	0.46404352	3.50E-19
<i>jag2b</i>	-1.12764153	0.457663287	7.77E-18
<i>notch1a</i>	-1.156345553	0.448647553	1.71E-26
<i>her12</i>	-1.268438471	0.415108831	2.19E-13
<i>dlb</i>	-1.315279555	0.401847621	1.51E-22
<i>dld</i>	-1.385814041	0.382673515	2.72E-21
<i>lfng</i>	-1.406649427	0.377186664	2.37E-18
<i>her2</i>	-1.822570493	0.282716797	2.91E-16

belonging to the *hes* and *her* families, and *lfng*^{203–205}—to name a few. In the nervous system, neurons and glial cells arise from the same set of neural progenitor cells. Absence of Notch signaling allows for the development of neurons and the presence of Notch signaling pushes the neural progenitor cells to form glial progenitors. Notch signaling continues to act on these glial progenitors and leads to the specification of OPCs (in the CNS) and Schwann cell progenitors (in the PNS). In the CNS, canonical Notch signaling mechanisms are known to suppress the OPC differentiation and myelination; however, non-canonical ligands (Contactin-1) will promote the development of mature myelinating neurons^{203,206–209}. In contrast, Notch signaling actively promotes the differentiation of mature Schwann cells and subsequent myelination in the PNS^{210,211}. The fact that genes involved in the Notch signaling process are down-regulated in *gas6* mutants, strongly suggest the involvement of *gas6* in glial lineage progression and formation of myelin. Furthermore, positive regulators of myelination like *egr2/krox20* (promotes differentiation of immature Schwann cells to produce myelin^{212,213}), and *mpz* (myelin protein zero: cell adhesion molecule involved in tight wrapping of myelin²¹⁴) are also down-regulated by at least 3-fold in *gas6* mutants; once again, indicating that *gas6* could most certainly be involved in gliogenesis and myelination.

Conclusion

Gene regulatory networks are inherently complex, and this has been demonstrated in a variety of developmental processes in several model organisms. In this study we successfully positioned 22 previously uncharacterized genes into the existing GRN governing caudal hindbrain formation in the zebrafish (Figure 4.1). Analysis of six mutant lines indicated that these genes are not absolutely required for r4-r6 formation but may have subtle roles. This leaves the previously reported factors RA, FGF, *hoxb1a*, *hoxb1b*, *hnf1ba* and *valentino* as key regulators of r4-r6 formation in the zebrafish. By extrapolation from work in the mouse ^{69,98,107}, it is likely that *krox20* and PG3 *hox* genes also play a role in r4-r6 development in the zebrafish. While this may seem to be a small number of essential genes, there are other GRNs that have a limited number of core regulatory factors, like that of the transcriptional network regulating ES cells. Biochemical and bioinformatic studies done in both mice and humans show that Oct4, Sox2 and Nanog are the master regulators controlling the pluripotency and self-renewal of embryonic stem cells. While there are other TFs involved in the larger embryonic stem cell GRN, they all feed into the core Oct4-Sox2-Nanog circuit ^{215,216}. In support of the complex nature of GRNs, we demonstrate that regulation of r4 and r5/r6 is achieved via different mechanism. Specifically, our results support a novel model wherein r4 genes are under the combinatorial regulation of RA and *hoxb1b*, whereas r5/r6 genes are downstream of the previously described RA, FGF *hnf1ba* and *valentino* factors.

We also identify novel interactions between the two gene-sets where the most striking observation is the repression of *gbx1* by *hoxb1a* in r4 (Figure 4.1). In conclusion, our study demonstrates the distinct mechanisms of gene regulation in r4 and r5/r6 which stands as evidence to the complex nature of the GRN governing caudal hindbrain development in the zebrafish.

APPENDIX A: 39 r4-restricted genes

r4 genes	Expression pattern	Reported hindbrain phenotype	Regulated by <i>hoxb1a</i>	<i>hoxb1b</i> RNA Seq	Probe Status
<i>dusp2</i>	r4	none			functional
<i>dusp6</i>	r4	none			functional
<i>egfl6</i>	r2/4/6	none			functional
<i>greb1l</i>	r2/4/6	none			functional
<i>spry1</i>	r4	none			functional
<i>hoxb2a</i>	r3 and weak in r4	none			functional
<i>meis1a</i>	r4	none			functional
<i>eng1b</i>	faint r4	none			functional
<i>fgf3</i>	r4	otic vesicle; (Mackereth 2005; Hans and Westerfield 2007)			functional
<i>irx7</i>	r3-5	<i>irx7</i> MO and <i>irx1b</i> MO shorten region from r1-4, (Stedman et al. 2009)			functional
<i>fgf8a</i>	r4	Shortened r3/5 (Jaszai 2003)		Up regulated	functional
<i>efnb2a</i>	r4	<i>efnb2a</i> MO and <i>epha4a</i> MO disrupt rhombomere boundaries, (Cooke, Kemp, and Moens 2005)			
<i>ccnjl</i>	moderate r1/2/4/7	none			poor signal
<i>sall4</i>	basal level in hindbrain, stronger in r4	none			poor signal
<i>col7a1l</i>	r4	none	X		did not work

<i>fabp7a</i>	middle part of r4	none	X		did not work
<i>slc1a2b</i>	faint r4	none	X		did not work
<i>btg2</i>	r4	none			did not work
<i>id2a</i>	very faint r4 staining	none			did not work
<i>sp8a</i>	r4	none			did not work
<i>cux2a</i>	r4	none			could not recover plasmid supplied by ZIRC
<i>ppp1r14ab</i>	r4	regulates <i>fgf3</i> in r4, (Choe et al. 2011)			did not work
<i>cyp26c1</i>	r2 to r4	otic vesicle, (Montalbano et al. 2016)			
<i>hoxb1b</i>	r4	r3/4 , (Weicksel et al. 2014; Zigman et al. 2014)			
<i>cyp26b1</i>	r3/4	r3/4/5, (Reijntjes, Rodaway, and Maden 2007)		Down regulated	
<i>prickle1b</i>	faint r4/ lateral neurons	r4/facial motor neuron, (Rohrschneider, Elsen, and Prince 2007)	X		
<i>hoxb1a</i>	r4	r4/hindbrain neurons, (Weicksel et al. 2014)			
<i>spry2</i>	r4	seen in neural plate, (Labalette et al. 2011)			
<i>epha4b</i>	very faint in r2/4/6	none			
<i>fgfr2</i>	dark hindbrain staining, stronger in r4	none			

<i>gfap</i>	whole hindbrain, r4 darker	none			
<i>kctd12.2</i>	r4	none			
<i>efnb3b</i>	r2/4/6	none			
<i>mecom</i>	r4	none	X		
<i>raraa</i>	ventral r4/entire hindbrain	none	X		
<i>lrrn1</i>	stronger in r4	none			
<i>nog2</i>	r2/4, not clear	none			
<i>znf503</i>	hindbrain r4/5/6	none			
<i>pax3b</i>	r4 (?)	none			

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APPENDIX B: 68 r5 and r6-restricted genes

r5/6 genes	Expression pattern	Reported hindbrain phenotype	hoxb1b RNA Seq	Probe Status
<i>zwi</i>	r3/5			did not work
<i>midn</i>	r3/5			did not work
<i>cellf2</i>	r5			functional
<i>gas6</i>	r5/6			functional
<i>gbx1</i>	throughout hindbrain except r4			functional
<i>mpz</i>	r3/5		Up regulated	functional
<i>nr2f2</i>	r2/3/5/6			functional
<i>sema3fb</i>	r3/5			functional
<i>tox3</i>	r3/5			functional
<i>col15a1b</i>	r5/6			functional
<i>bdnf</i>	faint in r3/5			
<i>bhlhe22</i>	maybe r5 or r6			
<i>cers2a</i>	faint in r5			
<i>crabp2a</i>	patches in r2/4, more on the ventral and dorsal plate, strong in r6			
<i>cygb1</i>	faint r3/5			
<i>dap1b</i>	very faint r3/5 staining			
<i>ddit4</i>	maybe in r3 and r5?			
<i>desma</i>	r3/5		Down regulated	
<i>dlst</i>	darker in r5/6			
<i>egr2a</i>	faint in r3/5			

<i>emilin2b</i>	dorsal part of r5			
<i>entpd1</i>	r3/5			
<i>ephb4a</i>	r2/5/6			
<i>flrt1b</i>	ventral r1 to r3, r5/6			
<i>flrt3</i>	maybe r4/5, not marked			
<i>hnf1bb</i>	r5/6			
<i>hoxa2b</i>	r2/3/5, faint in r4			
<i>hoxa3a</i>	r5/6			
<i>hoxb3a</i>	r5/6			
<i>irx3a</i>	r3/4/5 (?) not marked			
<i>irx4b</i>	r1/2/3/5			
<i>kdm5bb</i>	faint r5, strong in r6			
<i>mmp2</i>	r5			
<i>myo10l1</i>	r2/5			
<i>myo15aa</i>	r3/5			
<i>nab1a</i>	r3/5			
<i>nck2a</i>	r3/5			
<i>nkx2.2b</i>	r3/5			
<i>nrbf2b</i>	r3/5			
<i>ntn2</i>	partly in r4, dorsal side of rhombomere			
<i>nxn</i>	r3/5/7			
<i>prdx6</i>	dorsal r5/6			
<i>prox2</i>	extremely faint r5/6			
<i>rarab</i>	entire hindbrain, might be stronger in r5/6			
<i>rhoca</i>	weak staining in hindbrain with stronger in r3/5			
<i>schip1</i>	basal level throughout the			

	hindbrain, stronger in r3/5			
<i>sfxn1</i>	r6			
<i>sipa1l1</i>	r3/5			
<i>tead3a</i>	basal level in hinbrain, stronger in r3/5			
<i>tenm3</i>	faint staining in r3/5			
<i>uacab</i>	r3/5			
<i>wnt8b</i>	r3/5			
<i>znf536</i>	entire hindbrain, except r4			
<i>enc1</i>	r3/5			
<i>enc2</i>	faint r3/5			
<i>sema6dl</i>	faint staining in r3/5?			
<i>mafba</i>	r5/6	abducens, r5/6(Moens et al. 1996, 1998; Choe et al. 2008; Zannino, Sagerström, and Appel 2012; Ma, Grove, and Baker 2014)		
<i>mid1ip1l</i>	faint r5	embryonic development, (Eno, Solanki, and Pelegri 2016)		
<i>ca8</i>	r3/5	embryos development/neurons, (Aspatwar et al. 2013)		
<i>arrb2b</i>	r3 to r5	embryos development/somites, Li et al., 2015		
<i>lfng</i>	r2/4	facial motor neuron, (Nikolaou et al. 2009)		
<i>egr2b</i>	r3/5	hindbrain structural organization, (Miller et al. 2013)		

<i>coro1ca</i>	more in r3/r5; no very well defined	neural crest/pharyngeal arch 1&3, (Williamson et al. 2014)		
<i>egr1</i>	faint in r3/5	pharyngeal arch 3-7, (Dalcq et al. 2012; Zhang et al. 2013)		
<i>btbd6a</i>	r3/5	primary neurons, (Sobieszczuk et al. 2010)		
<i>epha4a</i>	r3/5	r3/5 boundary formation, (Cooke, Kemp, and Moens 2005; Van Hoecke et al. 2012)		
<i>hnf1ba</i>	r5/6/7	r5/6, (Sun and Hopkins 2001; Golling et al. 2002; Hans and Westerfield 2007; Nakamura et al. 2008; Chi et al. 2008)		
<i>ackr3b</i>	r3/5/6	trigeminal neurons, (Lewellis et al. 2013)		

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APPENDIX C: 85 hindbrain genes are potentially regulated by *hoxb1b*

Genes expressed in r4, r5, and r6	Up-regulated genes in <i>hoxb1b</i> RNA-Seq (n=7)	Down-regulated genes in <i>hoxb1b</i> RNA-Seq (n=78)	DE genes with specific rhombomere restricted expression pattern n=4
<i>atf3</i>		X	
<i>atp1a1a.2</i>		X	
<i>atp1a3a</i>		X	
<i>atp1a3b</i>		X	
<i>bsk146</i>		X	
<i>cacnb2a</i>		X	
<i>cacnb4b</i>		X	
<i>cadm2a</i>		X	
<i>calb2b</i>		X	
<i>cd81b</i>		X	
<i>cdh4</i>		X	
<i>cdx4</i>	X		
<i>col11a1b</i>		X	
<i>cyp1b1</i>	X		
<i>cyp26b1</i>		X	r3/4
<i>dab2</i>		X	
<i>dbh</i>		X	
<i>desma</i>		X	r3/5
<i>draxin</i>	X		

<i>drd2b</i>		X	
<i>egr3</i>		X	
<i>entpd3</i>		X	
<i>fez1</i>		X	
<i>fgf13a</i>		X	
<i>fgf8a</i>	X		r4
<i>fosab</i>		X	
<i>fsta</i>	X		
<i>gad1b</i>		X	
<i>gapdhs</i>		X	
<i>gata2b</i>		X	
<i>ghrb</i>		X	
<i>glrbb</i>		X	
<i>gpc1b</i>		X	
<i>gpm6aa</i>		X	
<i>gria2b</i>		X	
<i>gria3b</i>		X	
<i>grin1a</i>		X	
<i>homer1b</i>		X	
<i>hpdb</i>		X	
<i>hsp90aa1.2</i>		X	
<i>hsqb11</i>		X	
<i>hsqb9</i>		X	
<i>kif5aa</i>		X	
<i>lin7a</i>		X	

<i>mb</i>		X	
<i>mmp23bb</i>		X	
<i>mpz</i>	X		r3/5
<i>myhz1.1</i>		X	
<i>myt1la</i>		X	
<i>ndufa4</i>		X	
<i>nefma</i>		X	
<i>neurod6b</i>		X	
<i>npy8br</i>		X	
<i>olfm2a</i>		X	
<i>otpa</i>		X	
<i>pcsk1nl</i>		X	
<i>pdcd4b</i>		X	
<i>pdlim3b</i>		X	
<i>penka</i>		X	
<i>penkb</i>		X	
<i>pth1b</i>		X	
<i>pth2r</i>	X		
<i>ptn</i>		X	
<i>pvalb7</i>		X	
<i>rab11bb</i>		X	
<i>raly</i>		X	
<i>rgs7a</i>		X	
<i>rtn4r</i>		X	
<i>rxfp3.2b</i>		X	

<i>sardh</i>		X	
<i>scrt1a</i>		X	
<i>sgcd</i>		X	
<i>slc10a4</i>		X	
<i>slc6a1b</i>		X	
<i>sncgb</i>		X	
<i>sst1.1</i>		X	
<i>st8sia5</i>		X	
<i>stmn4l</i>		X	
<i>syng1a</i>		X	
<i>tfap2e</i>		X	
<i>tph2</i>		X	
<i>trpc4a</i>		X	
<i>trpm2</i>		X	
<i>tspan7b</i>		X	
<i>wnt4b</i>		X	

APPENDIX D: Protein sequences for *gas6* and *gbx1* CRISPR mutants

Protein sequences coded by *gas6* mutant alleles

WT	M R E L V W S F C L V L L C S G F C S P V S V S S	(1-25aa)
um296	M R W C G A S A S S C C V R A S A L P C P C P R A	(1-25aa)
um297	M R G C G A S A S S C C V R A S A L P C P C P R A	(1-25aa)
um298	M S W C G A S A S S C C V R A S A L P C P C P R A	(1-25aa)
um299	M S W C G A S A S S C C V R A S A L P C P C P R A	(1-25aa)
WT	R Q A H Q F L R R T R R A N Q V F E E T K Q G H L	(26-50aa)
um296	R P T S S C A E P A E P T R C S R R P N R A T W R	(26-50aa)
um297	R P T S S C A E P A E P T R C S R R P N R A T W R	(26-50aa)
um298	R P T S S C A E P A E P T R C S R R P N R A T W R	(26-50aa)
um299	R P T S S C A E P A E P T R C S R R P N R A T W R	(26-50aa)
WT	E R E C V E E K C T K E E A R E V F E N D P E T E	(51-75aa)
um296	G S V W R R S A L R R R R G K C S R M T R R R S I	(51-75aa)
um297	G S V W R R S A L R R R R G K C S R M T R R R S I	(51-75aa)
um298	G S V W R R S A L R R R R G K C S R M T R R R S I	(51-75aa)
um299	G S V W R R S A L R R R R G K C S R M T R R R S I	(51-75aa)
WT	Y F Y P K Y Q A C M E R F G D S E K K K Q	(76-96aa)
um296	S T P S I R L V W R D L G I Q R R R N R I Stop	(76-96aa)
um297	S T P S I R L V W R D L G I Q R R R N R I Stop	(76-96aa)
um298	S T P S I R L V W R D L G I Q R R R N R I Stop	(76-96aa)
um299	S T P S I R L V W R D L G I Q R R R N R I Stop	(76-96aa)
WT	D L I T C V H N I P D Q C S P N P C Y H Y G T V R C E D K K G E F R C H C F T G W S G A T C Q N D V D E C I S G N G G C E H V C N N T M G S Y K C S C E D G Y R L S G H H S C L D V D E C V E T P D V C G S A H C S N L I G G L E C L C D E G F I Y D N I S R S C V D V D E C E T H V C E E E C V N T P G S F R C F C D G R L G K R L S S D M R S C E S I S L D R P L D M R R N S R S L Y L G R M F S G I P V V R L R F R R R V Q T G F T A E F D L R T F D P E G V I F F A G G H L N S S W I V L L V H H G K L E L Q L K Y G V V S R V T S S G P Q V N D G Q W H K I S V E E Q G R S L V I K I D R E A V M K I A V N G D L F T L N K N M H E L N L T V G G V P F R D D G L V S R V N P R L D G C M K D W R W L T G E D T S I Q E T I R H N E R M Q C Y A V E D H S A F Y P G H G F A Y F N H S H G D N Q T L S V H V T L R A A S S M G V L F A L V R Q D R V P F S I S L S D Y H P G T L Q W T K A C T G V V G	(97-536aa)

Protein sequences coded by *gbx1* mutant alleles

WT	M Q R P S G T G T A F S I D S L I G T P Q P R P G (1-25aa)
um300	M Q R P S G T G T A F S I D S L I G T P Q P R P G (1-25aa)
um301	M Q R P S G T G T A F S I D S L I G T P Q P R P G (1-25aa)
WT	H L L Y T G Y P M F M P Y R P L M I P Q A L S H S (26-50aa)
um300	H L L Y T G Y P M F M P Y R P L M I P Q A L S H S (26-50aa)
um301	H L L Y T G Y P M F M P Y R P L M I P Q A L S H S (26-50aa)
WT	S L P S G I P P L A P L A S F A G R L T N T F C A (51-75aa)
um300	S L P S G I P P L A P L A S F A G R L T N T F C A (51-75aa)
um301	S L P S G I P P L A P L A S F A G R L T N T F C A (51-75aa)
WT	G L G Q G M P S M V A L T T T L P S F S D P P D S (76-100aa)
um300	G L G Q G M P S M V A L T T T L P S F S D P I F N (76-100aa)
um301	G L G Q G M P S M V A L T T T L P S F S D P P Stop (76-98aa)
WT	F Y P P Q E M P G P R L G A D G T G M N (101-120aa)
um300	C X G V A L E I V S T P R R R C R D P G Stop (101-120aa)
WT	R Q E S P H D E L K G S E L L N F T E T F Q A V A G E T K L Y S S D D E K L D L K A A E A A C S D R E D S S A D S E N E S F S D G N T C A S A S Q K G K L K G G S Q D A L P P G G S A G K S R R R R T A F T S E Q L L E L E K E F H C K K Y L S L T E R S Q I A H A L K L S E V Q V K I W F Q N R R A K W K R I K A G N V N N R S G E P V R N P K I V V P I P V H V N R F A V R S Q H Q Q I E P G S R P (121-316aa)

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